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13. ABSTRACT (Maximum 200 Words)

Based on Preliminary Data, we hypothesized that loss of retinoic acid receptor function might promote dysregulated growth and loss of epithelial polarity. We now report that retinoids and the retinoic acid receptor-beta are important regulators of proliferation and polarity normal mammary epithelial cells through modulation of 1) the CREB-binding protein, CBP and 2) laminin 5 expression. As proposed in Objective 1A, and described in the BODY of this report, we have been successful in identifying a retinoid-regulated gene, the CREB-binding protein, CBP whose function is important in mammary epithelial cell growth regulation and polarity. CBP is a known critical regulator of retinoid-signaling, however, the role of CBP in regulating growth and epithelial cell polarity has been previously unknown. As proposed in Objective 1B, we have suppressed CBP function in mammary epithelial cells and demonstrated that suppression of CBP function results in loss of growth regulation and polarity. We also provide evidence that CBP functions in a positive feed-back loop with retinoids and the retinoic acid receptor-beta 2. In the final year of this grant we have shown that CBP regulates polarity and growth through modulation of laminin-5 expression. These findings provide important insight into how retinoid and retinoic acid receptors may act to regulate normal mammary epithelial cell growth and polarity. These findings also can be rapidly translatated to provide 1) biomarkers for breast cancer risk and 2) response to chemoprevention.

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INTRODUCTION:
Retinoids are important for the normal growth and differentiation of epithelial cells. We hypothesize that loss of retinoic acid receptor function might be an important early event in breast cancer carcinogenesis. In Preliminary Data we demonstrated that retinoids are important regulators of proliferation and may promote a more differentiated phenotype in normal mammary epithelial cells. To investigate the hypothesis that loss of normal retinoic acid receptor function might promote carcinogenesis of HMECs, we developed an *in vitro* system in which retinoic acid receptor function is suppressed by expression of a dominant-negative retinoic acid receptor (DNRAR). We observe that HMECs expressing the DNRAR exhibit dysregulated growth and do not exhibit normal structural differentiation *in vitro*. These studies suggest that loss of retinoic acid receptor function in HMECs might result in loss of normal cellular differentiation and thereby may promote mammary carcinogenesis. We used this model system to isolate and characterize retinoid-regulated genes that may be important in the normal growth and differentiation of human mammary epithelial cells. As described in the BODY of this report, we have been successful in identifying a retinoid-regulated gene, the CREB-binding protein, CBP. CBP is a known critical regulator of retinoid-signaling, however, the role of CBP in regulating growth and epithelial cell polarity has been previously unknown. In **Year 2** of this grant, we deomonstrated that CBP functions in a positive feed-back loop with retinoids and the retinoic acid receptor-beta 2. These findings provide important insight into how retinoid and retinoic acid receptors may act to regulate normal mammary epithelial cell growth and polarity. In **Year 3** we demonstrated that CBP regulates proliferation and polarity through modulation of laminin-5 expression. Theses are novel findings that we are currently translating to develop novel markers for assessing breast cancer risk and response to prevention agents.

BODY:
Objective I: Isolation and characterization of retinoid-responsive genes activated or suppressed by retinoic acid receptors in normal human mammary epithelial cells.
Formation of a polarized epithelial ductal structure is a characteristic of normal mammary cells and serves to distinguish normal from malignant mammary epithelial cells. We observe that inhibition of retinoic acid receptors in normal human mammary epithelial cells (HMECs) by a dominant-negative retinoic acid receptor (DNRAR) results in dysregulated growth and an inability of cells to form a normal polarized ductal epithelial structure when cultured in 3-dimensional extracellular matrix culture.

A) Isolation of retinoid-regulated genes: We utilized the techniques of "differential display" and differential gene expression studies (gene "chip") to isolate retinoid-regulated genes regulated in normal human mammary epithelial cells (HMECs) that may be critical for mammary epithelial cell polarity and growth regulation. Several candidate genes were identified. Differential gene expression studies were correlated with detailed cytogenetic results in extracellular matrix-resistant and retinoid-resistant mammary epithelial cells (Seewaldt et al. Journal of Cell Biology, 2001, and Dietze et al., resubmitted to Journal of Cell Biology, 2003, see appendix).

Cytogenetic analysis of extracellular matxix-resistant and retinoid-resistant HMECs demonstrated frequent chromosomal loss of 16p (74%) or rearrangement at 16p13 (16%)

(Seewaldt et al. Journal of Cell Biology, 2001, see appendix). **The high frequency of 16p loss in rECM-resistant HMECs suggested that 16p harbors a gene whose loss may be important in resistance to both retinoid and extracellular matrix.** Chromosomal loss at 16p13 is observed in a majority of benign and malignant papillary neoplasms of the breast and loss of 16p is frequently observed in premalignant breast lesions such as atypical hyperplasia. Chromosome 16p13.3 is the location of the human CREB binding protein (CBP), a nuclear protein important for retinoid-signaling, growth regulation, and apoptosis.

Consistent with our cytogenetic data, we observe that **resistance to extracellular matrix-induced apoptosis is associated with a 75% *decrease* in expression of CBP** (Dietze et al. Microscopy Research and Techniques, 2002; Troch et al. BBRC, 2003, see appendix). CBP is a tightly regulated transcription factor that plays a critical regulatory role as an integrator of diverse signaling pathways including those mediated by retinoids, estrogen, and calcium. Given the important role that CBP plays in apoptosis, growth regulation, and retinoid-signaling, we hypothesize that loss of CBP expression may promote loss of extracellular matrix-regulated growth regulation and polarity.

B) Characterization: *Confirming the importance of CBP in regulating polarity and growth regulation in HMECs:* The function of our candidate gene, CBP, in HMECs was examined using sense and anti-sense sequences cloned into either retroviral or inducible expression vectors. HMECs with suppressed CBP were grown in prepared extracellular matrix and analyzed for growth characteristics and the ability to form a normal epithelial ductal structure in prepared extracellular matrix. We observed that suppression of CBP resulted in loss of epithelial cell polarity and growth regulation. Loss of growth regulation was associated with loss of laminin-5 expression and alpha3/beta1 integrin signaling (Dietze et al. Journal of Cell Biology, re-submitted 2003, see appendix).

Demonstration that CBP is regulated by retinoids and retinoic acid receptors. While it is well established that CBP is a co-activators of retinoid-signaling, the transcriptional regulation of CBP is uncharacterized. We tested whether all-*trans*-retinoic acid and the retinoic acid receptor-beta 2 induced the expression of CBP mRNA and protein in mammary epithelial cells (Dietze et al. Microscopy Research and Techniques, 2002, Troch et al. Cancer Research, submitted, 2002, see appendix). We identified that retinoids and the retinoic acid receptor-beta 2 regulate the expression of CBP protein. We then analyzed the promoter of CBP and identified putative retinoic acid receptor response elements in the promoter of CBP. This is an important finding because this suggests that retinoids, retinoic acid receptor-beta 2, and CBP can function in a positive-feed back loop that promotes retinoid-sensitivity in HMECs.

C) Characterization of CBP regulated mammary epithelial cell polarity through regulation of laminin-5 expression.

We further analyzed our differential gene expression data to identify further down-stream targets of CBP that may play a critical role in retinoid-regulation of mammary epithelial cell growth and polarity.

Laminin-5 expression is decreased in prepared extracellular matrix- (rECM) resistant

HMECs. We previously observed that sensitivity to rECM in HMECs required polarized expression of α3/β1-integrin. Differential gene expression studies, semi-quantitative RT-PCR, and Western analysis were performed to test whether the loss of sensitivity to rECM correlated with altered expression of laminin-5 and/or α3/β1-integrin mRNA. Differential gene expression studies demonstrated decreased expression of all three laminin-5 chains (α3, β3, and γ2) in rECM-resistant, CBP-"poor" HMECs relative to HMEC controls with normal CBP expression. Semi-quantitative RT-PCR confirmed a 98% decrease in laminin-5 α3-chain (p≤0.01), an 88% decrease in laminin-5 β3-chain (p≤0.01), and a 75% decrease in laminin-5 γ2-chain (p≤0.01) mRNA expression relative to early passage controls. There was no significant change in the level of α3/β1-integrin mRNA expression. Western analysis similarly demonstrated an 85% (p<0.001) decrease in laminin-5 α3-chain protein expression in rECM-resistant HMECs relative to rECM-sensitive HMEC controls. There was a 130% (p<0.002) decrease in laminin-5 α3-chain protein in rECM-senstivite HMEC controls relative to rECM-resistant HMECs. These observations demonstrate that the presence of rECM-resistance in HMECs correlates with a loss of laminin-5 mRNA and protein expression.

Lack of polarized expression of laminin-α3 and integrin-α3 proteins in rECM-resistant HMECs cells grown in rECM. rECM-sensitive HMECs controls (with normal CBP expression) and rECM-resistant cells (CBP-"poor") were grown in rECM and tested for 1) laminin-5 α3-chain and 2) α3- and β1-integrin expression by immunohistochemistry (clones P5H10, P1F2, and P4C10, respectively). rECM-sensitive cells exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins. In contrast, rECM-resistant, CBP-"poor" HMECs grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of both laminin-5 α3-chain and α3-integrin. As predicted by differential gene expression studies and Western analysis, there was also a qualitative decrease in laminin-5 α3-chain expression in late passage HMECs relative to controls. rECM-resistant HMECs grown in rECM exhibited polarized basal β1-integrin expression but had an increase in the amount of cytosolic expression relative to early passage cells. These observations demonstrate a loss of polarized expression of laminin-5 α3-chain and α3-integrin in rECM-resistant HMECs.

Suppression of CBP expression in HMECs alters both laminin-α3 and integrin-α3 protein expression in rECM culture. We observed that rECM-resistant HMECs grown in rECM culture exhibit 1) reduced levels of CBP protein expression and 2) disorganized expression of both laminin-5 α3-chain and α3-integrin. This observation led us to hypothesize that suppression of CBP in HMECs would alter laminin-5 α3-chain and α3-integrin expression and/or distribution. CBP protein expression was suppressed in rECM-sensitive HMECs by treatment with CBP-specific, antisense ODN (A99424V). HMECs with suppressed CBP expression exhibited disorganized plasma membrane and cytosolic distribution of laminin-5 α3-chain and α3-integrin. β1-integrin expression was observed at the basal surface. In contrast, HMECs treated with inactive CBP ODN (scrA99424V) exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins . These observations demonstrate that suppression of CBP protein expression in HMECs alters the distribution of both laminin-5 α3-chain and α3-integrin.

Decreased CBP expression in rECM-resistant HMECs correlates with decreased LAMA3A

promoter activity. We tested whether the observed decrease in CBP and laminin-5 α3-chain expression in rECM-resistant HMECs correlated with decreased LAMA3A promoter activity. rECM-resistant HMECs and rECM-sensitive HMEC controls were transiently transfected with a CAT reporter coupled to the *LAMA3A* promoter sequence (1403 bp, GenBank Accession Number AF279435) and grown in rECM culture. rECM-resistant, HMECs with decreased CBP and laminin-5 α3-chain expression exhibited a 91% decrease in *LAMA3A* promoter activity relative to rECM-sensitive HMECs (p≤0.01). These experiments demonstrate in HMECs a positive correlation between 1) the level of CBP and laminin-5 α3-chain protein expression and 2) *LAMA3A* promoter activity.

LAMA3A promoter activity in HMECs with suppressed CBP protein expression. We next tested whether suppression of CBP protein expression resulted in decreased *LAMA3A* promoter activity. LAMA3A-CAT reporter activity was compared in rECM-sensitive HMECs treated with either CBP-specific antisense ODNs (A33423V) or inactive ODNs (scrA33423V). A 92% decrease (p<0.01) in *LAMA3A* promoter activity was observed in rECM-sensitive HMECs grown in rECM and treated with CBP-specific ODNs (A33423V) relative to cells treated with inactive ODNs (scrA33423V). No significant difference in *LAMA3A* promoter activity was observed in HMECs treated with or without inactive ODNs. These observations demonstrate that suppression of CBP expression in HMECs results in a reduction in *LAMA3A* promoter activity.

Lack of CBP occupancy of the human laminin 5 (LAMA3A) promoter correlates with rECM-resistance. The human *LAMA3A* promoter contains three AP-1 sites at positions −387, −185, and −127. The AP-1 site, at position −185, has been previously shown to be critical for basal activity in mammary epithelial cells. Chromatin immunopreciptation (ChIP) was performed in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells and controls to test whether the observed 1) decrease in laminin-5 α3-chain expression and 2) loss of *LAMA3A* activity correlated with a lack of CBP binding to the 277 bp AP-1-"rich" site of the *LAMA3A* promoter (position -402 to −125). rECM-sensitive HMECs grown in rECM demonstrated CBP binding to the AP-1-"rich" site of the *LAMA3A promoter.* In contrast, rECM-resistant HMECs, with decreased CBP and laminin-5 α3-chain expression, failed to demonstrate CBP binding. These observations suggest that a decrease in CBP expression might promote loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter.

Suppression of CBP expression in HMECs results in loss of CBP occupancy of the LAMA3A promoter. rECM-sensitive HMECs were treated with active CBP ODNs and tested by ChIP to determine whether suppression of CBP protein expression resulted a loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Apoptosis-sensitive HMECs treated with CBP-specific ODNs, and grown in rECM, did not demonstrate CBP occupancy of the *LAMA3A* promoter. In contrast, HMECs, treated with inactive ODNs, and grown in rECM demonstrated CBP-occupancy (Figure 10 b). These observations demonstrate that suppression of CBP expression in HMECs by antisense ODNs results in a loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter. Since the AP-1 site, at position −185, is critical for basal activity in mammary epithelial cells, these observations provide a mechanism by which loss of CBP expression might promote loss of *LAMA3A* promoter activity and laminin-5 α3-chain

expression in HMECs.

KEY RESEARCH ACCOMPLISHMENTS:

A) **Development of HMECs resistant to retinoid and extracellular matrix-growth regulation, polarity, and apoptosis.**

B) **Identification of the CREB-binding protein, CBP, as a potential regulator of retinoids and extracellular-matrix signaling.**

C) **Demonstration that suppression of CBP protein expression by antisense oligonucleotides blocks the induction of extracellular matrix-growth regulation and -polarity in HMECs.**

D) **Demonstration that retinoids and the retinoic acid receptor-beta 2 mediate transcription of CBP.**

E) **Demonstrate that CBP regulates extracellular matrix-growth regulation, polarity, and apoptosis through modulation of laminin 5 mRNA and protein.**

F) **Demonstration that binding of CBP to the laminin 5 promoter is critical for laminin 5 expression.**

Reportable Outcomes:
Publications:

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., and Caldwell, L.E. Suppression of p53 in human mammary epithelial cells increases sensitivity to extracellular matrix-induced apoptosis. J. Cell Biol., 155: 471-486, 2001.

2) Dietze, E.C. Mrózek, K., Caldwell, L.E., Yokoyama, K., Srinivasan, K., Hobbs, K.B., and **Seewaldt, V.L.** CBP modulates epithelial polarity and resistance to extracellular matrix-induced apoptosis in p53(-) human mammary epithelial cells. Reubmitted to J. Cell Biol, 2003.

3) Dietze, E.C., Caldwell, L.E., Marcom, K., Collins, S.J., Swisshelm, K., Hobbs, K.B., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of CBP/p300. Microscopy Res. and Tech., 59:23-40, 2002.

4) Dietze, E.C., Srinivasan, K., Hobbs, K.B., Rieffler, M.B., and **Seewaldt, V.L.** Regulation of CBP/p300 expression by all-*trans*-retinoic acid and RARβ2. BBRC, 2003.

Abstracts and Presentations:

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., Dietze, E., and Caldwell, L.E. Suppression of p53 in Human Mammary Epithelial Cells increases sensitivity to extracellular matrix-induced apoptosis. Loss of p53 function promotes sensitivity to extracellular matrix-induced apoptosis in human mammary epithelial cells. Keystone Symposia, Banff, Alberta, Canada, Feb 10, 2002.

2) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., Dietze, E., Hobbs, K.B., and Caldwell, L.E. Suppression of p53 in Human Mammary Epithelial Cells increases sensitivity to extracellular matrix-induced apoptosis. CBP modulates resistance to extracellular

matrix-induced apoptosis in human mammary epithelial cells. AACR, San Francisco, CA. April 10, 2002.

3) Dietze, E.C., Mrozek, K., Caldwell, L.E., Hobbs, K.B., Yokoyama, K., and **Seewaldt, V.L.** CBP modulates resistance to extracellular matrix-induced apoptosis in human mammary epithelial cells. Gordon Conference Mammary Carcinogenesis, Tuscany, Italy, April 2, 2002. Invited oral presentation.

4) Dietze, E.C., Mrozek, K., Caldwell, L.E., Hobbs, K.B., Yokoyama, K., and **Seewaldt, V.L.** CBP/p300 and extracellular matrix-induced apoptosis in human mammary epithelial cells. 18th IUC International Cancer Congress, June 30, 2002, Oslo, Norway.

5) Troch, M., Bowie, M., Dietze, E.C., and **Seewaldt, V.L.** CBP/p300 expression is regulated by a retinoic acid response element. AACR Workshop in Prevention, October 16, 2002, Boston, MA.

6) Troch, M., Bowie, M., Dietze, E.C., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate CBP/p300 expression through a retinoic acid response element. San Antonio Breast Conference, December 13, 2002, San Antonio, TX.

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1) Duke University Assistant Professorship 1/00 (Seewaldt)
2) Promoted to Associate Professor 11/01 (Seewaldt)

Training Supported by this Award:
Svetlana Grupin, Duke Undergraduate Student
Katherine Hobbs, Duke Undergraduate Student

Gregory Bean, Duke Undergraduate student
Brooke Ratliff, Postdoctoral Fellow

Conclusions:

We have determined that 1) CBP and laminin 5 are critical mediators of extracellular matrix regulated-growth and polarity and that 2) retinoids and the retinoic acid receptor-beta 2 regulate CBP transcription and polarized expression of laminin 5. **These observations have important implications:** Currently, we are rapidly developing tools to identify women at high-risk for developing breast cancer. While this new technology holds a great deal of promise, our ability to label women as "high-risk" is rapidly outstripping our ability to develop prevention strategies. In preclinical models we identified a critical role for CBP, in maintaining normal mammary homeostasis. CBP is important for retinoid-, estrogen-, calcium-, and COX-2 signaling. **The development of *in vitro* models of CBP loss and mammary hyperplasia will be extremely valuable for the testing of a wide variety of prevention strategies for breast cancer. Information gained from this proposal can be immediately translated to 1) predict breast cancer risk and 2) develop and assess response to prevention agents.** Furthermore, the laminin 5 promoter has been found to be methylated in lung cancer. These observations in combination with observations in this proposal set the stage for testing for laminin 5 promoter methylation in early precancerous breast lesions.

References:

None.

Appendices

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., and Caldwell, L.E. Suppression of p53 in human mammary epithelial cells increases sensitivity to extracellular matrix-induced apoptosis. J. Cell Biol., 155: 471-486, 2001.

2) Dietze, E.C. Mrózek, K., Caldwell, L.E., Yokoyama, K., Srinivasan, K., Hobbs, K.B., and **Seewaldt, V.L.** CBP modulates epithelial polarity and resistance to extracellular matrix-induced apoptosis in p53(-) human mammary epithelial cells. Submitted to J. Cell Biol.

3) Dietze, E.C., Caldwell, L.E., Marcom, K., Collins, S.J., Swisshelm, K., Hobbs, K.B., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of CBP/p300. Microscopy Res. and Tech., 59:23-40, 2002.

4) Dietze, E.C., Srinivasan, K., Hobbs, K.B., Rieffler, M.B., and **Seewaldt, V.L.** Regulation of CBP/p300 expression by all-*trans*-retinoic acid and RARβ2. BBRC, 2003.

Retinoids and Retinoic Acid Receptors Regulate Growth Arrest and Apoptosis in Human Mammary Epithelial Cells and Modulate Expression of CBP/p300

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ABSTRACT   Retinoids and retinoic acid receptors (RARs) are important mediators of normal epithelial cell homeostasis. To assess the role of retinoids and RARs in regulating growth arrest and apoptosis in benign and malignant mammary epithelial cells, two model systems were developed: 1) RAR function was suppressed in retinoid-sensitive normal human mammary epithelial cells (HMECs) by the dominant-negative retinoic acid receptor, RARα403 (DNRAR), and 2) retinoid-resistant MCF-7 breast cancer cells were transduced with a functional RARβ2. Inhibition of RAR function by the DNRAR in HMECs resulted in retinoid-resistance, increased proliferation, and dysregulated growth when cells were cultured in reconstituted extracellular matrix (rECM). Expression of RARβ2 in MCF-7 cells resulted in sensitivity to retinoid-induced growth arrest and apoptosis. The CREB-binding protein (CBP) and the homologous protein p300 are tightly regulated, rate-limiting integrators of diverse signaling pathways and are recruited during retinoid-mediated transcriptional activation. The relationship between retinoid receptor expression, growth regulation, and transcriptional regulation of CBP/p300 is poorly understood. Inhibition of RAR function in HMECs by DNRAR suppressed expression of CBP/p300 and expression of RARβ2 in MCF-7 cells promoted induction of CBP/p300 when cells were treated with 1.0 μM all-*trans*-retinoic acid (ATRA). These results suggest that ATRA and RARs regulate growth arrest of HMECs and modulate CBP/p300 protein expression. Since CBP and p300 are normally present in limiting amounts, their regulation by ATRA and RARs may be an important element in the control of transcriptional activation of genes regulating growth arrest and apoptosis. *Microsc. Res. Tech. 59: 23–40, 2002.*   © 2002 Wiley-Liss, Inc.

INTRODUCTION

Vitamin A (retinol) and its derivatives (retinoids) are important for normal cellular growth and differentiation (Hong et al., 1997). The actions of retinoids are ultimately thought to be mediated through specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors (Mangelsdorf et al., 1994). Multiple retinoic acid receptors have been identified; among these are RARα, β, and γ. RARα is expressed ubiquitously in adult tissue and RARγ is primarily expressed in skin. RARβ is unique because it is primarily expressed in epithelial cells and exhibits induced expression in response to retinoic acid (ATRA) that is mediated by an enhancer element, the retinoic acid response element (RARE), within its promoter (DeThe et al., 1990; Hoffmann et al., 1990). While normal cultured human mammary epithelial cells (HMECs) express RARβ mRNA, most breast cancer cell lines fail to express this gene (Swisshelm et al., 1994; Seewaldt et al., 1995, 1997a). Furthermore, RARβ mRNA expression is upregulated in senescent

HMECs, suggesting that this gene may be involved in regulating terminal differentiation in mammary epithelial tissue (Swisshelm et al., 1994).

Retinoids have also been found to be important for the prevention of certain cancers. Retinoids can halt the progression of disease in premalignant lesions of the oral cavity, cervix, and skin and are effective in preventing the development of second primary tumors of the aerodigestive tract and lung (Lippmann et al., 1993; Volkes et al., 1993). There is also evidence that retinoids may be important for the prevention of breast cancer. The risk of breast cancer is increased for

women with a lower dietary intake of vitamin A and beta carotene but not for women with dietary deficiencies of vitamin C or E (Hunter et al., 1993).

The potential association between vitamin A deficiency and the development of cancer suggests that retinoid-dependent signaling pathways have a role in the suppression of carcinogenesis. Selective loss of expression of RARβ has been observed in ductal carcinoma in situ and in a majority of breast cancers, suggesting that loss of RARβ function may be an important early event in breast cancer development (Widschwendter et al., 1997; Xu et al., 1997). Furthermore, loss of heterozygosity at the chromosomal region 3p22-25 containing the coding sequence for the RARβ is the most frequent chromosomal aberration observed in morphologically normal lobules adjacent to breast cancer (Deng et al., 1996). This suggests that in a subset of sporadic breast cancer a tumor suppressor gene at 3p22-25 may be important in initiation of early progression of mammary tumorigenesis (Deng et al., 1996). Recently, an alternative mechanism for the development of retinoid-resistance has been observed in breast cancer cells (Sommer et al., 1999). RARβ4 is an alternatively spliced product of the RARβ P2 promoter that may function as a naturally occurring dominant-negative repressor of RAR-mediated growth suppression (Nagpal et al., 1992) and promote loss of mammary epithelial polarity (Berard et al., 1994). Five-fold increased expression of RARβ4 has been detected in breast cancer cell lines relative to HMECs (Sommer et al., 1999). These observations suggest that there may be multiple mechanisms to account for the development of retinoid-resistance in mammary epithelial cells.

One group of transcriptional cofactors that may be recruited by RARs is the CREB-binding protein (CBP)/p300 family which plays a critical role in integrating multiple signal transduction pathways by the induction of selective gene transcription (Yao et al., 1998; Robyr et al., 2000). CBP and p300 have been shown to be required for growth arrest and apoptosis (Vo et al., 2001). Retinoids are important mediators of growth arrest in epithelial cells and CBP and p300 are transcriptional coactivators of the RAR family of nuclear receptors (Kawasaki et al., 1998; Yao et al., 1998). This raises the possibility that the regulation of gene expression by RARs could be in part mediated by the availability of CBP and p300. In the work presented here, we examine the role of retinoids and retinoic acid

receptors in mammary epithelial cell growth regulation, apoptosis, and the regulation of CBP/p300 expression.

ALL-*TRANS*-RETINOIC ACID AND RETINOIC ACID RECEPTORS MEDIATE GROWTH ARREST IN NORMAL AND MALIGNANT MAMMARY EPITHELIAL CELLS

Retinoids play a critical role in epithelial cell homeostasis and the loss of retinoid receptor function is felt to be an important event in mammary carcinogenesis. We developed two experimental systems to test the hypothesis that retinoids and RARs are critical mediators of growth arrest and apoptosis in mammary epithelial cells. In the first experimental system, a dominant-negative approach was utilized to suppress retinoic acid receptor function in HMECs. In the second system, a functional RAR-β2 was expressed in breast cancer cell lines that fail to express RAR-β2 and are resistant to the growth inhibitory effects of all-*trans*-retinoic acid (ATRA).

ATRA Mediates Growth Arrest in HMECs

HMECs express RAR-α, RAR-β2, and RAR-γ and are sensitive to the growth inhibitory effects of ATRA (Swisshelm et al., 1994; Seewaldt et al., 1995, 1997a). Growth inhibition is independent of p53 expression (Seewaldt et al., 1999b). We and others previously identified that ATRA regulates cyclin D1 levels by post-translational proteolysis (Langenfield et al., 1997; Seewaldt et al., 1997b) and that retroviral-mediated over-expression of cyclin D1 inhibits ATRA-mediated growth arrest and differentiation in HMECs (Seewaldt et al., 1999a). The cyclin D1 promoter does not contain either a retinoic acid response element (RARE) or an AP-1 site. It is felt that the primary target of ATRA during the induction of growth arrest is the regulation of ubiquitin-mediated proteolysis of cyclin D1 through recognition of the cyclin D1 C-terminal PEST sequence (Langenfield et al., 1997; Seewaldt et al., 1999a). Cyclin D1/cdk4 complexes are thought to play a major role in phosphorylating the retinoblastoma protein (pRB) during G₁-phase (Kato et al., 1993). Recent studies suggest that pRB is the downstream target of cyclin D1, as suppression of hyperphosphorylated pRB (ppRB) inhibits ATRA-mediated growth arrest (Dietze et al., 2000). These observations suggest that ATRA mediates growth inhibition in HMECs by a cyclin D1/pRB-mediated pathway.

Suppression of RAR Function in HMECs

A truncated RAR exhibiting dominant-negative activity was utilized to inhibit RAR function in HMECs to test whether inhibition of RAR function results in loss of ATRA-mediated growth inhibition. Dominant-negative retinoic acid receptors have been used to unravel the physiologic function of retinoids in several cell types (Tsai et al., 1992, 1993, 1994; Anderson et al., 1995; Imakado et al., 1995; Saitou et al., 1995). For this study we utilized a dominant-negative mutant of RARα (RARα403) driven by a retroviral LTR (DNRAR) (Tsai et al., 1993). This RARα derivative is a negative transcriptional regulator that can simultaneously block all wild-type RAR isoforms (−α, −β, and −γ) (Damm et al., 1993). This truncated mutant RAR retains the abil-

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Abbreviations

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ATRA	all-*trans*-retinoic acid
RAR	retinoic acid receptor
RARβ2	retinoic acid receptor-beta isoform-2
RARE	retinoic acid response element
HMEC	normal human mammary epithelial cell
DNRAR	dominant-negative retinoic acid receptor-α403
ECM	extracellular matrix
rECM	reconstituted extracellular matrix
CBP	CREB-binding protein
Cdk	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
TdT	terminal deoxynucleotidyl transferase
pRB	retinoblastoma protein
CAT	chloramphenicol acetyl transferase

A.

B.
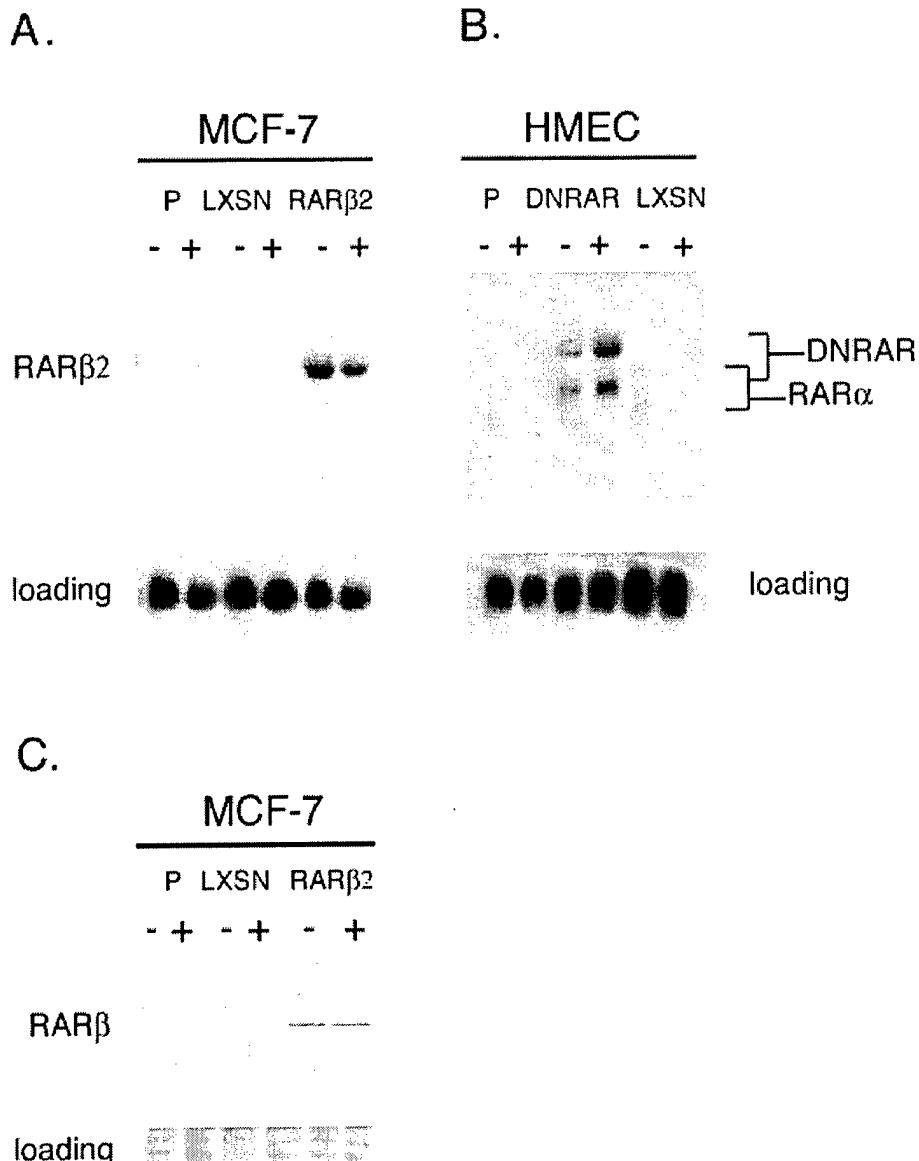
Fig. 1. Expression of transduced constructs in normal and malignant mammary epithelial cells. **A:** Expression of exogenous RARβ2 mRNA in MCF-7 breast cancer cells. Northern analysis of MCF7-P parental cells (P), MCF7-RARβ2 transduced breast cancer cell lines (RARβ2), and vector controls (LXSN) treated for 48 hours without (−) or with (+) 1.0 μM ATRA. Ten μg of total RNA were loaded per lane. 36B4 served as a loading control. **B:** Expression of the RARα403 DNRAR construct in HMECs. Northern analysis was performed on RNA extracted from HMEC-P parental cells (P), from HMEC-DNRAR cells infected with LRARα403SN retroviral vector (DNRAR), and from HMEC-LXSN vector control cells (LXSN). Cells were treated without (−) or with (+) 1.0 μM ATRA for 48 hours. The blot was probed with a 1.3 kb (SmaI) RARα cDNA fragment. Ten μg of total RNA were loaded per lane. 36B4 serves as a loading control. **C:** RARβ protein expression in transduced MCF-7 breast cancer cells. Western blot of MCF7-RARβ2 cells (RARβ2), MCF7-P parental cells (P), and MCF7-LXSN vector controls (LXSN) treated without (−) or with (+) 1.0 μM ATRA for 48 hours. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. One hundred μg of protein lysate were loaded per lane. The blot was probed with an RARβ-specific polyclonal antiserum (Seewaldt et al., 1995). Unidentified 45 kd and 65 kd protein bands were used as a loading control.

ity to dimerize with RXR and bind retinoic acid response elements, suggesting that the DNRAR acts by forming transcriptionally inactive heterodimers that compete for DNA binding with the natural RAR-RXR heterodimers (Damm et al., 1993). Control cells were infected by the control LXSN retroviral vector (without insert).

Expression of the transduced RARα403 DNRAR construct was confirmed by Northern analysis of total cellular RNA extracted from both HMEC-LXSN vector controls and HMEC-DNRAR cell lines utilizing an RARα probe. As expected, HMEC-LXSN demonstrated expression of endogenous RARα mRNA (Fig. 1B). Expression of the transduced DNRAR insert was observed

A

Micromoles all-*trans*-retinoic acid

B

Micromoles all-*trans*-retinoic acid
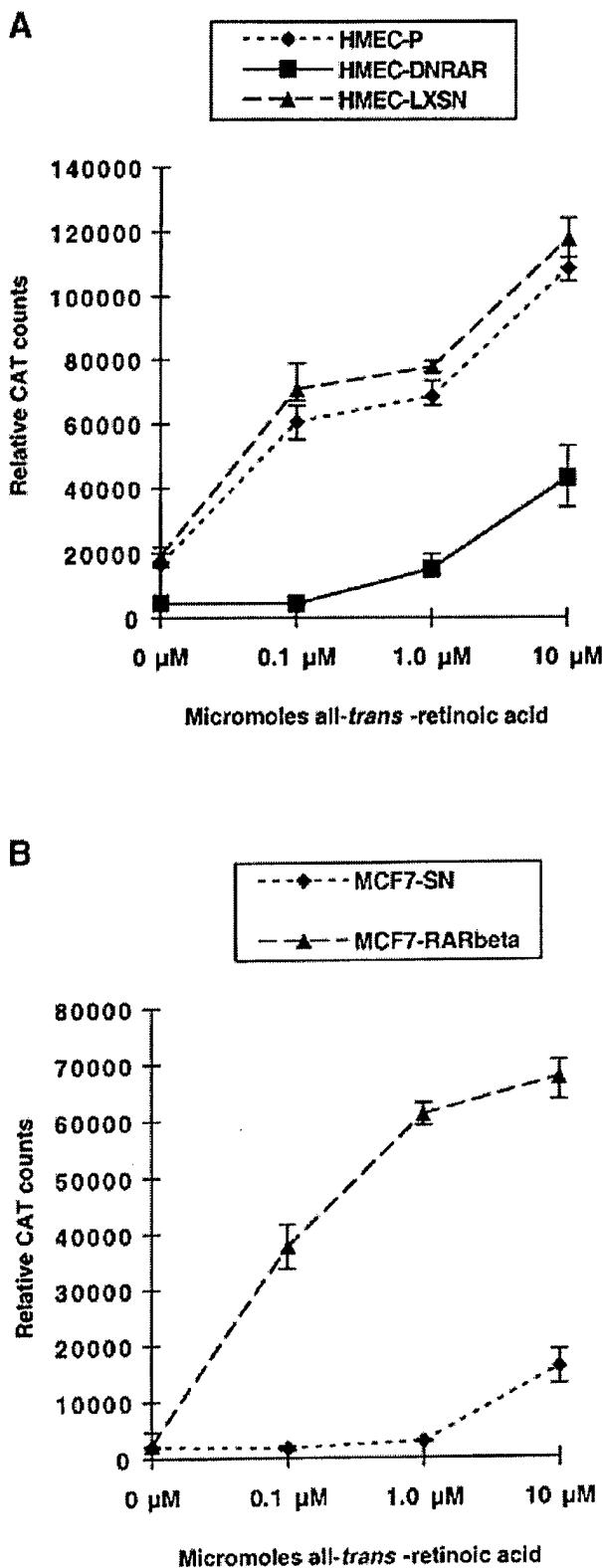
in HMEC-DNRAR cells at >10-fold higher than the endogenous RARα (Fig. 1B). As previously observed, both a 4.8- and a 3.0-kb DNRAR transcript were observed (Tsai et al., 1993).

Expression of RARβ2 in Breast Cancer Cells

To test the role of RARβ2 in mediating breast cancer cell growth arrest we constructed an LXSN-based retroviral vector harboring the coding sequence of the human RARβ2 gene. This vector includes a 94 bp sequence of 5' untranslated adenosine deaminase sequence to place the cDNA in an optimal translational context (Seewaldt et al., 1995), as well as the neomycin phosphotransferase selectable marker. This RARβ retroviral construct is designated LaRARβ2SN. The breast cancer cell line MCF-7 does not express RARβ2 mRNA and is relatively resistant to the growth inhibitory effects of ATRA at concentrations less than 5 μM (Swisshelm et al., 1994). MCF-7 cells were infected with the LaRARβ2SN retroviral vector and G418 resistant cells were isolated. These transductants were designated MCF7-RARβ2. Vector control cells were obtained by infecting MCF-7 with the retroviral vector LXSN without insert and were designated MCF7-LXSN. Experiments depicted in the following figures were performed on MCF7-RARβ2-clone-9 and MCF7-LXSN-clone-2 and are representative of experiments performed in 5 RARβ2- and 3 LXSN-transduced MCF7 clones. Northern and Western blots were performed on viral-transduced MCF-7 cells to determine the levels of RARβ2 mRNA and protein expression, respectively. The LTR-initiated 4.8-kb RARβ2 mRNA was observed in MCF7-RARβ2 cells but not in MCF7-P parental cells nor in MCF7-LXSN controls (Fig. 1A). Endogenous RARβ2 mRNA was not expressed by MCF7-LXSN or MCF7-P parental cells (Fig. 1A), nor was it reactivated by the expression of the exogenous RARβ2. RARβ protein expression was determined by Western blotting using an RARβ-specific polyclonal antiserum. The expected single 53-kd band was detected in cellular lysate from MCF7-RARβ2 transduced clones but not in the lysate of MCF7-LXSN controls or MCF7-P cells (Fig. 1C).

Modulation of RAR Function in Benign and Malignant Mammary Epithelial Cells Alters ATRA-Induced RARE Activity

DNRAR, RARα403, blocks ATRA-stimulated transcription both by binding ATRA and competing directly

Fig. 2. Modulation of RAR function and expression alters ATRA-induced RARE activity. **A:** DNRAR construct LRARα403SN inhibited ATRA-mediated *trans*-activation of βRARE in HMEC-DNRAR cells (passage 12–14) relative to HMEC-P parental cells (passage 12–14) and HMEC-LXSN vector controls (passage 12–14). **B:** Expression of RARβ2 in retinoid-resistant MCF-7 cells increased ATRA-mediated *trans*-activation relative to MCF7-P parental cells and HMEC-LXSN vector controls. Cells were transfected with pRRE4-tkCAT plasmid, treated with 0, 0.1, 1.0, or 10 μM ATRA for 24 hours and cell lysates were assayed for CAT reporter activity. pCMV-GH was used as a transfection efficiency control. CAT counts were corrected for growth hormone activity and total protein (Seewaldt et al., 1997a). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. Data represent an average of three independent transfections performed in duplicate.

A HMEC-LXSN

B MCF7-LXSN

HMEC-DNRAR

MCF7-RARβ2
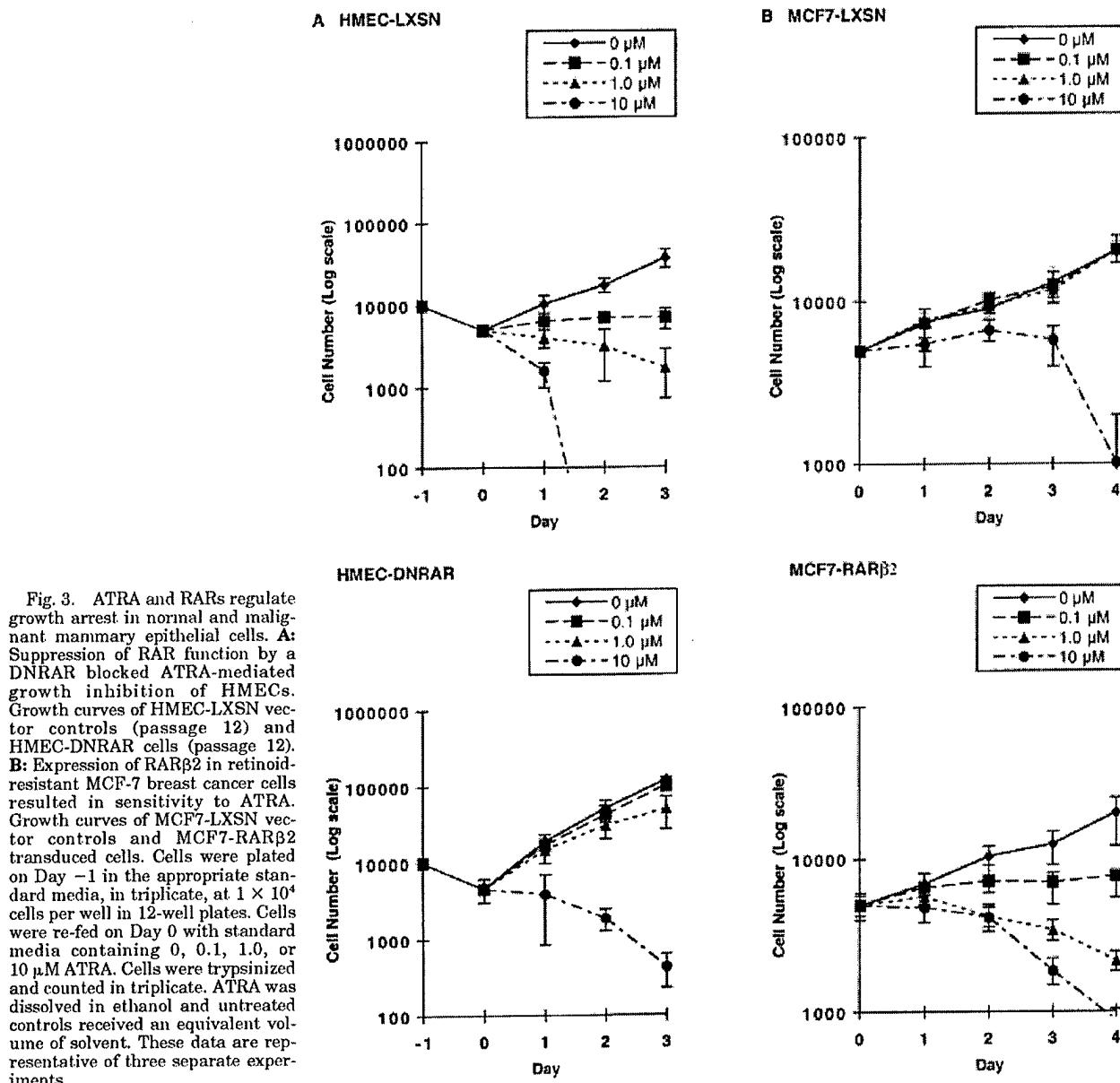
Fig. 3. ATRA and RARs regulate growth arrest in normal and malignant mammary epithelial cells. A: Suppression of RAR function by a DNRAR blocked ATRA-mediated growth inhibition of HMECs. Growth curves of HMEC-LXSN vector controls (passage 12) and HMEC-DNRAR cells (passage 12). B: Expression of RARβ2 in retinoid-resistant MCF-7 breast cancer cells resulted in sensitivity to ATRA. Growth curves of MCF7-LXSN vector controls and MCF7-RARβ2 transduced cells. Cells were plated on Day −1 in the appropriate standard media, in triplicate, at 1×10^4 cells per well in 12-well plates. Cells were re-fed on Day 0 with standard media containing 0, 0.1, 1.0, or 10 μM ATRA. Cells were trypsinized and counted in triplicate. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

TABLE 1. Percentage distribution of cells in G_1- and S-phase in ATRA-treated cells

Cell Type	ATRA (M)	Day 0	Day 1	Day 2	Day 3	Day 4
HMEC-LXSN	10^{-7}	60/20	60/21	63/15	65/12	67/11
	10^{-6}	60/20	60/19	65/11	73/03	79/02
HMEC-DNRAR	10^{-7}	55/30	53/31	54/30	57/30	58/29
	10^{-6}	55/30	56/30	57/29	63/25	69/22
MCF7-LXSN	10^{-6}	61/20	62/19	61/21	60/18	60/21
MCF7-RARβ2	10^{-6}	63/16	68/10	68/10	70/03	73/01

Adherent HMEC-LXSN vector controls, HMEC-DNRAR transduced cells, MCF7-LXSN vector controls, and MCF7-RARβ2 transduced cells were treated for 0 to 4 days with 0.1 μM or 1.0 μM ATRA. The percentage of cells in G_1- and S-phase of the cell cycle were determined by flow cytometry and recorded in tabular form (%G_1-/%S-phase). ATRA was dissolved in ethanol and Day 0 controls received an equivalent volume of solvent. These data are representative of three separate experiments.

for endogenous RARs to form inactive heterodimers that compete with active dimers for the RARE. DNRAR- and LXSN-transduced HMECs were not subcloned and all experiments were performed on mass culture. To confirm dominant-negative activity of the DNRAR construct in HMECs, loss of sensitivity to ATRA was tested by transient expression of chloramphenicol acetyl transferase (CAT) driven by a RARE

A.
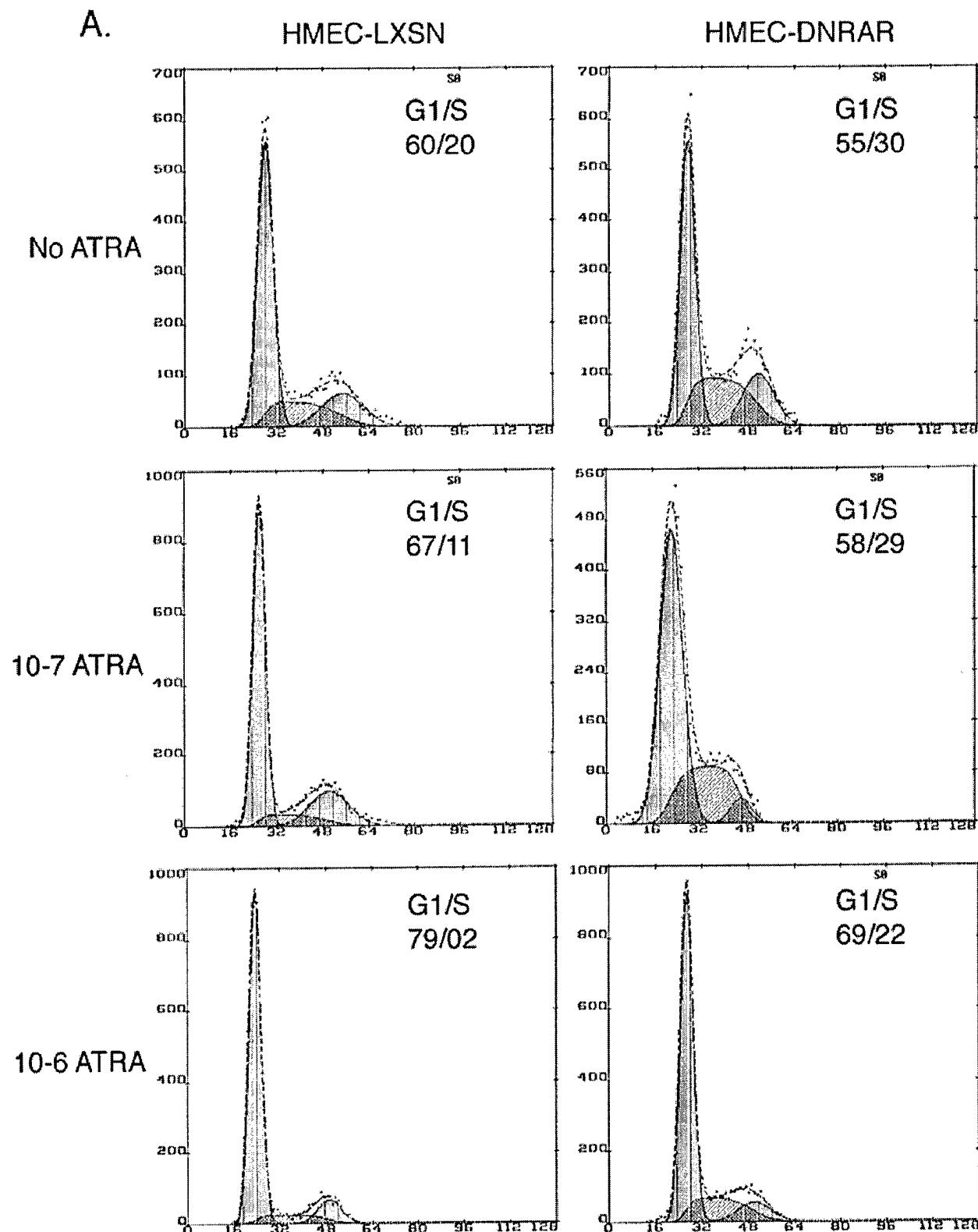
Fig. 4. Modulation of RAR function and expression alters the cell cycle distribution of normal and malignant mammary epithelial cells. **A:** Cell cycle analysis of HMEC-LXSN vector controls (passage 12) and HMEC-DNRAR cells (passage 12) treated with 0, 0.1, or 1.0 μM ATRA and **(B)** MCF7-LXSN vector controls and MCF7-RARβ2 cells treated with 0 or 1.0 μM ATRA for 4 days. Cells were trypsinized and harvested for cell cycle analysis on Day 4. The percentage of cells in G_1 and S-phase of the cell cycle was determined by flow cytometry. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

B.

MCF7-LXSN   MCF7-RARβ2

No ATRA

G1/S
61/20

G1/S
63/16

10-6 ATRA

G1/S
60/21

G1/S
73/1
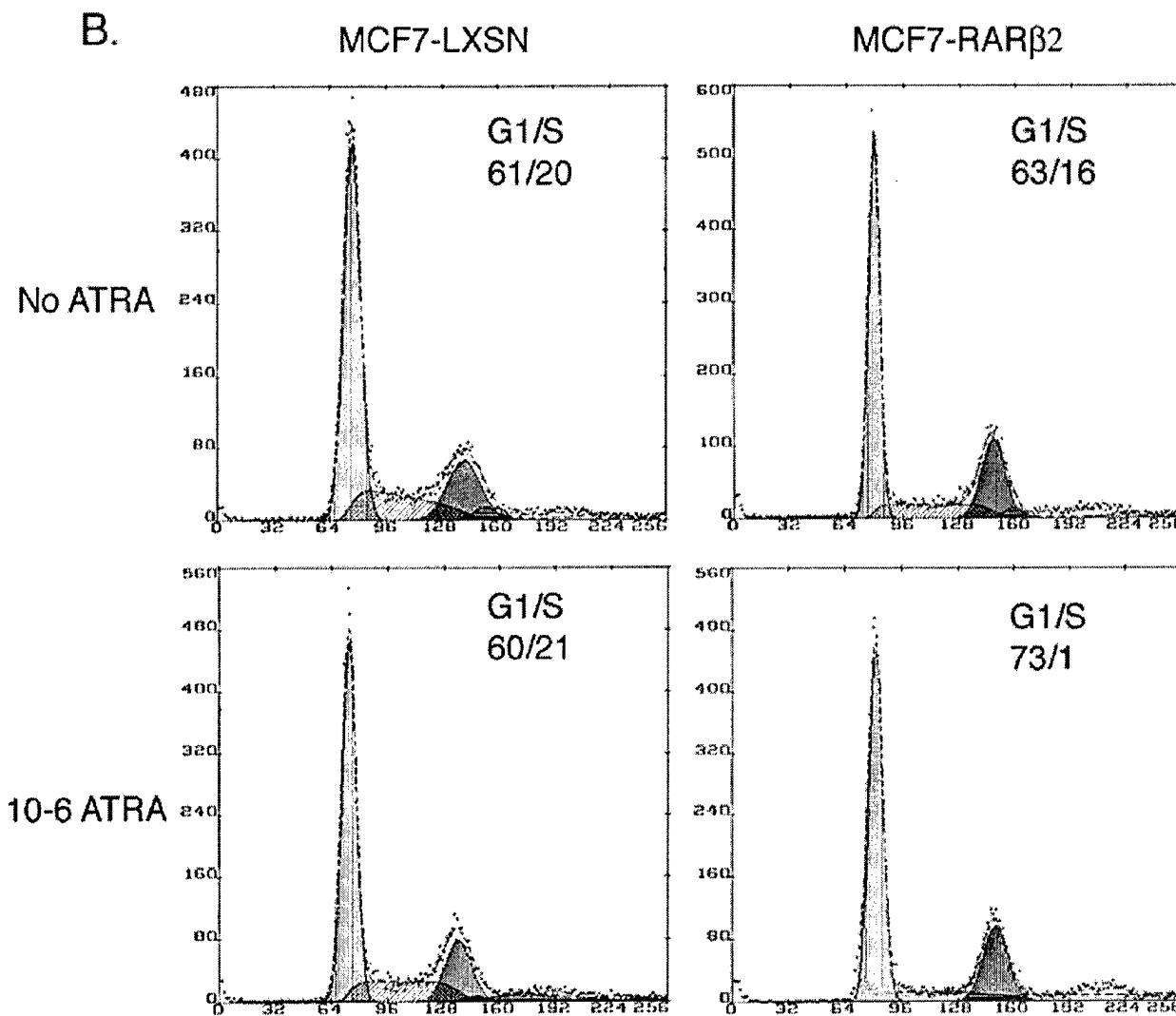
Figure 4.   (Continued.)

sequence (Seewaldt et al., 1997b). HMEC-LXSN and HMEC-P parent cells treated with 0.1–10 μM ATRA exhibited a 3–6-fold increase in RARE-CAT activity relative to untreated cells (Fig. 2A). In contrast, HMEC-DNRAR cells were resistant to ATRA-mediated *trans*-activation of the RARE at concentrations of 0.1–1.0 μM ATRA and only showed partial induction at concentrations of 10 μM ATRA (Fig. 2A). This observed inhibition of ATRA-mediated *trans*-activation in the DNRAR expressing HMECs relative to controls demonstrates the dominant-negative activity of our construct in HMECs and is consistent with previous observations utilizing this construct (Tsai et al., 1993). At 10 μM ATRA there was only partial suppression of ATRA-mediated *trans*-activation, indicating that the dominant-negative activity of this construct could be overcome by increasing concentrations of ATRA. These data demonstrate the ability of the DNRAR to inhibit normal RAR function in HMECs.

The ability of ATRA to stimulate RARE-mediated transcription was tested in retinoid-resistant MCF7-LXSN vector controls and MCF7-RARβ2 transduced cells. A 15–30-fold increase in RARE activity was detected in MCF7-RARβ2 cells treated with 0.1–10 μM ATRA (Fig. 2B). In contrast, increased RARE activity was not detected in MCF7-LXSN controls treated with 0.1 and 1.0 μM ATRA, but an 8-fold increase was detected after treatment with 10 μM ATRA (Fig. 2B). These observations suggest that expression of RARβ2 in retinoid-resistant MCF-7 breast cancer cell lines increases the ability of ATRA to induce RARE-mediated transcription.

ATRA and RARs Regulate Proliferation of Mammary Epithelial Cells

Surprisingly, untreated HMEC-DNRAR cells (passage 10–15) exhibited a significantly decreased doubling time relative to untreated HMEC-LXSN vector

controls and HMEC-P parental cells at the same passage number (Fig. 3A). In cultures passaged in parallel, we observed that HMEC-DNRAR cells had a 12–18-hour doubling time while HMEC-LXSN vector controls (passage 12) and HMEC-P parental cells exhibited a 24-hour doubling time (data not shown). These observations demonstrate that suppression of RAR function by a dominant-negative approach results in dysregulated proliferation in HMECs (Seewaldt et al., 1997a). The precise molecular mechanism by which inhibition of RAR function results in increased proliferation in untreated HMECs is currently an area of investigation in our laboratory.

Expression of the DNRAR resulted in resistance to the growth-inhibitory effects of ATRA. Increased growth inhibition of HMEC-LXSN controls was observed with increasing concentrations of ATRA and increasing time of exposure (Fig. 3A, Table 1). HMEC-DNRAR cells were resistant to the growth-inhibitory effects of ATRA relative to controls. Growth of HMEC-DNRAR cells was inhibited at 10 μM ATRA, suggesting that either the block could be overcome at higher concentrations of ATRA or that cells experienced a direct cytotoxic effect (Fig. 3A). This shifting of the dose–response curve to ATRA is evidence that the DNRAR has dominant-negative activity in HMECs. These data also demonstrate that ATRA inhibits the proliferation of HMECs in culture and that inhibition of RAR function blocks this growth inhibition.

MCF7-LXSN vector control cells were relatively resistant to the growth-inhibitory effects of 1.0 μM ATRA (Fig. 3B). Retinoid-resistance was overcome at 10 μM ATRA. In contrast, MCF7-RARβ2 transduced cells exhibited growth inhibition when treated with 0.1–1.0 μM ATRA. Increased growth inhibition was observed with increasing concentrations of ATRA (Fig. 3B, Table 1). These data provide evidence that expression of a functional RARβ2 in retinoid-resistant MCF-7 breast cancer cells results in increased sensitivity to the antiproliferative effects of ATRA. The importance of RARβ2 in regulating growth inhibition is supported by observations made by other investigators (Liu et al., 1996; Sun et al., 2000)

Modulation of RAR Function in Benign and Malignant Mammary Epithelial Cells Results in Altered Cell Cycle Distribution

To further investigate the ability of RARs to regulate growth arrest of HMECs, flow cytometric (FACS) analysis was performed on HMEC-DNRAR cells (passage 13–14) and HMEC-LXSN vector controls (passage 13–14) (Fig. 4A, Table 1). Analysis of isolated nuclei stained with propidium iodide demonstrated that untreated HMEC-DNRAR cells had a 50 ± 5% increase in the percentage of cells in S-phase relative to untreated HMEC-LXSN vector control cells (Fig. 4A). This is consistent with the observation that HMEC-DNRAR cells have an increased rate of proliferation relative to HMEC-LXSN controls.

HMECs whose normal RAR function was inhibited by the DNRAR were relatively resistant to the ATRA-mediated G_1 block observed in vector control cells: HMEC-LXSN treated with 0, 0.1, and 1.0 μM ATRA for 4 days had 20%, 11%, and 2% of cells in

S-phase, respectively (Fig. 4A, Table 1). In contrast, 30%, 29%, and 22% of HMEC-DNRAR cells treated with 0, 0.1, and 1.0 μM ATRA, respectively, for 4 days were in S-phase (Fig. 4A, Table 1). These results suggest that RARs are important mediators of ATRA-mediated growth inhibition and that suppression of RAR function in HMECs results in a significant increased percentage of cells in S-phase relative to controls.

The ability of RARβ2 to suppress proliferation in the breast cancer cell line MCF-7 was then tested. FACS analysis was performed on MCF7-RARβ2 transduced cells and MCF7-LXSN controls treated with 0 and 1.0 μM ATRA to further investigate the effect of RARβ2 expression on MCF-7 cell cycle progression. MCF7-RARβ2 cells treated with 1.0 μM ATRA for 4 days exhibited a 16 ± 3% increase in the percentage of cells in G_1 and a 94 ± 5% decrease in the percentage of cells in S-phase (Fig. 4B, Table 1). In contrast, MCF7-LXSN vector control cells did not exhibit a decrease in the percentage of cells in S-phase when treated with 1.0 μM ATRA for 4 days (Fig. 4B, Table 1). This cell cycle analysis suggests a critical role for ATRA and RARβ2 in mediating the G_1/S-phase distribution of MCF-7 cells.

RETINOIDS AND RETINOIC ACID RECEPTORS MEDIATE APOPTOSIS IN MALIGNANT BUT NOT IN NORMAL MAMMARY EPITHELIAL CELLS

There is evidence that retinoid receptors may be important mediators of apoptosis. Apoptosis plays an important role in embryogenesis, in normal tissue involution, and in regulating the death of terminally differentiated cells (Evans et al., 1998). There is evidence that retinoid-mediated truncation defects of the embryonic limb are the result of RAR-β2-mediated apoptosis (Soprano et al., 1994). Tumor response to therapy may depend on the ability of individual malignant cells to undergo apoptosis (Evans et al., 1998).

In addition to the above-noted growth arrest, MCF7-RARβ2 transduced cells underwent morphologic changes consistent with apoptosis beginning 4 days after treatment with 1.0 μM ATRA. These changes included nuclear condensation, loss of adherence, and cell shrinkage (Fig. 5). ATRA-treated MCF7-LXSN cells and untreated MCF7-RARβ2 transduced cells did not exhibit evidence of apoptosis (Fig. 5). Untreated MCF7-RARβ2 transduced cells exhibited modest cytoplasmic shrinkage relative to vector controls (Fig. 5), likely reflecting the presence of endogenous retinoids present in the fetal calf serum used in the cell culture medium.

The terminal deoxynucleotidyl transferase (TdT) method was also used to detect apoptotic strand breaks. In this assay, 3′ hydroxyl termini of apoptotic-induced strand breaks are labeled with biotin-dUTP by exogenous TdT and can be detected in situ or by flow cytometry after staining with avidin-conjugated fluorescein isothiocyanate (Seewaldt et al., 1997a). Incorporation of labeled dUTP was seen in MCF7-RARβ2 transduced cells treated with 1.0 μM ATRA for 6 days. This was not observed in untreated MCF7-LXSN and
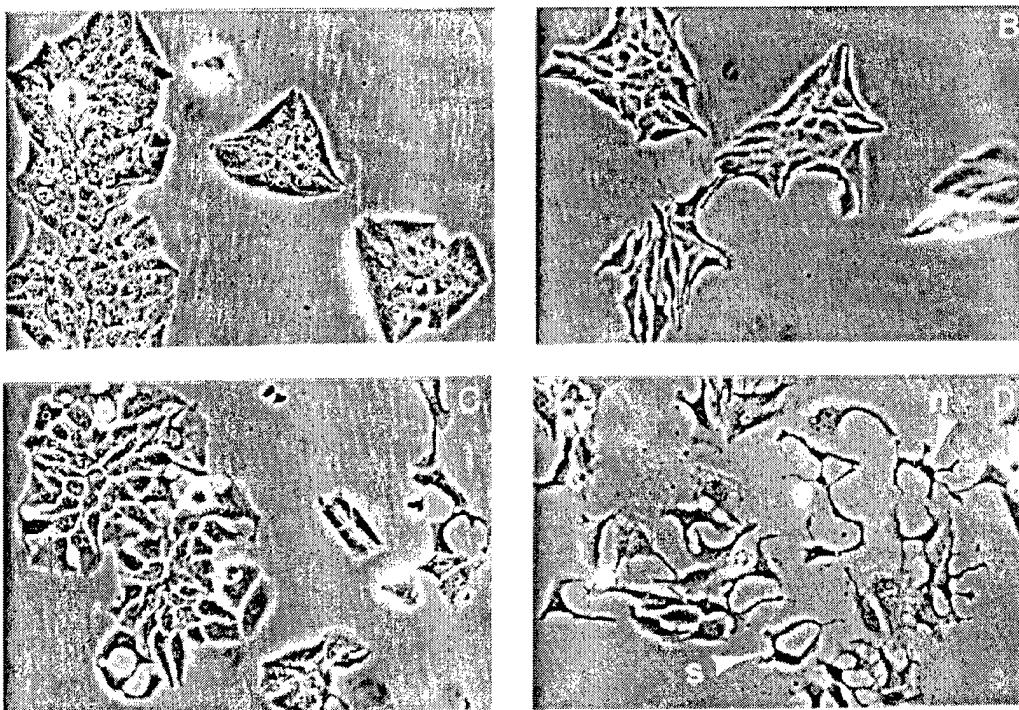
Fig. 5. Morphologic effects of retinoic acid on RARβ2 transduced MCF-7 cells and vector controls. Representative MCF7-LXSN vector controls (A,C) and MCF7-RARβ2 transduced cells (B,D) were incubated with (C,D) and without (A,B) 1.0 μM ATRA for 6 days. MCF7-RARβ2 transduced cells treated with ATRA exhibited nuclear condensation (n) and cell shrinkage (s). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent.

MCF7-RARβ2 controls or ATRA-treated MCF7-LXSN vector cells (Fig. 6). Similar results have been subsequently observed by other investigators (Liu et al., 1996). Taken together, these observations indicated that ATRA and RARβ2 mediate apoptosis in the breast cancer cell line MCF-7.

In order to determine whether ATRA mediates apoptosis in HMECs, we treated HMECs with ATRA and tested for apoptosis by morphologic criteria and by biochemical parameters. HMECs treated with 1.0 μM or 10 μM ATRA and examined by electron microscopy did not exhibit morphologic changes characteristic of apoptosis (data not shown). Internucleosomal DNA fragmentation is characteristic of apoptosis and distinguishes it from other modes of cell death such as necrosis (Holtz et al., 1992; Tepper et al., 1992). Similarly, we did not observe increased fragmented cytoplasmic DNA by the diphenylamine assay 2, 4, or 6 days after treatment of HMECs with 1.0 μM ATRA or 10 μM (data not shown). Moreover, ethidium bromide-stained DNA extracted from ATRA-treated HMECs did not demonstrate DNA laddering following 2, 4, or 6 days treatment with 1.0 μM ATRA (data not shown). Finally, apoptotic strand breaks were not detected by TdT staining in HMECs after 5 days treatment with 1.0 μM ATRA (data not shown). These data together suggest that while ATRA induces growth arrest in HMECs, ATRA does not induce apoptosis in HMECs.

INHIBITION OF RETINOID RECEPTOR FUNCTION IN NORMAL MAMMARY EPITHELIAL CELLS BLOCKS GROWTH REGULATION AND POLARITY BY RECONSTITUTED EXTRACELLULAR MATRIX (rECM)

Interaction between basement membrane and mammary epithelial cells is thought to play an important role in regulating growth arrest and epithelial polarity (Folkman et al., 1978; Petersen et al., 1992; Alford et al., 1998). There is evidence that these normal interactions are disrupted early during mammary carcinogenesis. While HMECs undergo growth arrest in culture in the presence of rECM, breast cancer cells and established breast cancer cell lines fail to exhibit similar growth arrest (Petersen et al., 1992). Thus, it is hypothesized that interaction with basement membrane may serve to distinguish the growth patterns of normal and malignant mammary epithelial cells.

To test the role of retinoid receptors in ECM-induced growth arrest and polarity, HMEC-LXSN vector controls and HMEC-DNRAR transduced cells were grown in rECM as a single cell suspension. HMEC-LXSN vector controls grew exponentially until Day 6 and then growth arrested on Day 7 (Fig. 7) forming a uniform population of spherical colonies. Mean diameter of these colonies at Day 9 was 23.5 μ (±10 μ) (Fig. 7). In contrast, HMEC-DNRAR transduced cells continued to proliferate within rECM beyond the time point when
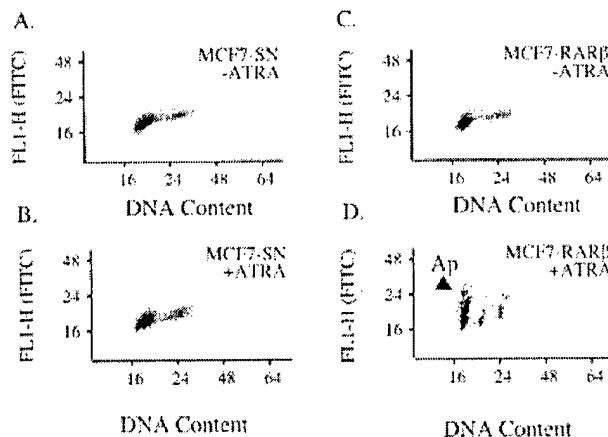
Fig. 6. Fluorescence labeling of apoptotic strand breaks by the TdT method in ATRA-treated MCF-7 cells. Representative MCF7-RARβ2 transduced cells (C,D) and MCF7-LXSN vector controls (A,B) treated with (B,D) or without (A,C) 1.0 μM ATRA for 6 days. Cells were incubated with TdT in the presence of biotin-labeled dUTP (Boehringer Mannheim). Detection was with avidin-conjugated fluorescein isothiocyanate (Seewaldt et al., 1995). MCF7-RARβ2 transduced cells treated with ATRA demonstrated apoptotic strand breaks (ap). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent.


Fig. 7. Inhibiting RAR receptor activity by a DNRAR enhances the proliferation of HMECs cultured in rECM. The mean diameter of spheres formed by HMEC-LXSN vector controls (passage 10) and HMEC-DNRAR cells (passage 10) whose retinoid receptor function is inhibited by a DNRAR is plotted as a function of days in culture. Cells were plated in rECM on Day 0 and the diameter of growing spherical cell colonies was measured with an eyepiece equipped with micrometer spindle. For both HMEC-LXSN vector controls and HMEC-DNRAR transduced cells, the 20 largest colonies were measured at each time point.

vector control cells underwent growth arrest (Fig. 7). Moreover, in contrast with control cells, HMEC-DNRAR transduced cells formed large, dense, irregular colonies. The mean diameter of these colonies was 62 μ (±10 μ) at Day 9, which was significantly larger than that of HMEC-LXSN vector control cells (Fig. 7). These data suggest that inhibition of RAR function in HMECs may result in loss of growth inhibition by rECM.

We next tested whether loss of retinoid receptor function could inhibit the formation of a normal epithelial ductal structure in vitro. Light micrographs of toluidine blue-stained, Epon-embedded, thick sections of HMEC-LXSN vector control cells cultured for 14 days in rECM demonstrated regular, spherical colonies (Fig. 8A,B). Electron micrographs show that HMEC-LXSN control cells grew in organized, acinus-like structures consisting of a single layer of epithelial cells, connected by numerous desmosomes, organized around a central lumen (Fig. 9A,B).

A fundamental property of normal epithelial cells is their ability to organize into polarized structures manifested by the characteristic location of intracellular organelles. When HMEC-LXSN control cells were cultured in rECM and examined by electron microscopy, the resulting acinus-like structure exhibited cellular organization typical of normal polarized epithelium: 1) microvilli were primarily distributed on the luminal and lateral surfaces but not on the basal surface, 2) secretory vacuoles were present on the luminal surface and not on the basal surface, and 3) mitochondria were primarily located at the basal surface (Fig. 9A,B).

In contrast, light micrographs of HMEC-DNRAR transduced cells grown in rECM for 14 days exhibited large, dense, irregularly shaped multilayered clusters of cells (Fig. 8C,D). Unlike normal control cells, the surface of these cell clusters projected irregularly into the surrounding rECM. Electron micrographs demonstrated disorganized clusters of cells with an absence of normal epithelial polarity (Fig. 9C,D). Evidence of lack of polarity included: 1) cells were not organized into spherical, single layered structures; 2) a lumen was not present; 3) microvilli were present on all cell surfaces; and 4) secretory vacuoles and mitochondria were randomly distributed throughout the cytoplasm. These data suggest that suppression of RAR function results in increased proliferation and inhibits the ability of HMECs to form a polarized ductal epithelial structure in rECM. Our results suggest that ATRA and retinoic acid receptors may play an important role in rECM-signal transduction critical for maintaining a normal mammary epithelial phenotype and loss of RAR function may thereby promote mammary carcinogenesis.

EXPRESSION OF RARβ2 IN BREAST CANCER CELLS PROMOTES GROWTH REGULATION BY RECM AND INDUCTION OF APOPTOSIS

We tested whether expression of RARβ2 in MCF-7 breast cancer cells might inhibit proliferation in rECM. Both MCF7-RARβ2 and MCF7-LXSN cells were cul-
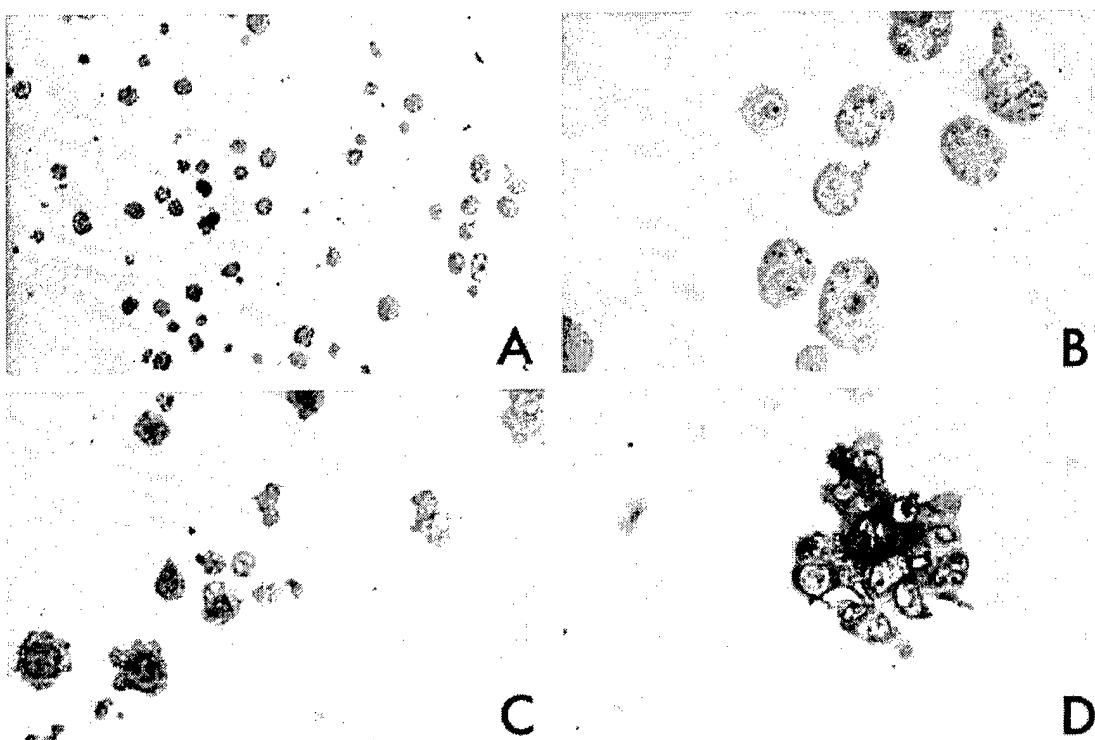
Fig. 8. Morphologic appearance of HMECs with DNRAR mediated suppression of RAR function grown in rECM. **A,B:** HMEC-LXSN vector control cells grown in rECM for 14 days. Cells form acinus-like spherical colonies that underwent growth arrest after 7 to 8 days in culture. Vector control cells demonstrate regular, spherical colonies surrounding a central lumen with sharply delineated boundaries, consistent with differentiated mammary glandular epithelium. **C,D:**

HMEC-DNRAR transduced cells whose retinoid receptor was inhibited by a DNRAR grown in rECM for 14 days. Cells formed large, dense irregularly shaped multicellular colonies with no central lumen and did not undergo growth arrest after 14 days in culture. Magnification of **A,C** = ×10 objective; magnification of **B,D** = ×40 objective. Epon-embedded 1 μ sections were stained with toluidine blue.

tured in prepared rECM as a single cell suspension. MCF7-LXSN vector control cells grew exponentially in rECM and formed large, dense, irregular colonies. Mean diameter of these colonies at Day 9 was 39 μ (±5.0 μ) (Fig. 10). In contrast, MCF7-RARβ2 transduced cells grew exponentially until Day 5 and then exhibited a progressively reduced growth rate (Fig. 10). The mean diameter of these colonies on Day 9 was 22 μ (±4.0 μ), which was significantly lower than that of MCF7-LXSN vector control cells (Fig. 10). These data suggest that expression of RARβ2 function in retinoid-resistant MCF-7 breast cancer cells may promote growth inhibition by rECM.

To investigate the potential mechanism for the observed reduction in proliferation in rECM, 20 MCF7-RARβ2 cell clusters were surveyed by electron microscopy on Day 5. Nineteen clusters (95%) exhibited morphologic evidence of apoptosis by the following criteria: 1) cell shrinkage, 2) margination of chromatin, and 3) the presence of apoptotic bodies (Fig. 11). In contrast, MCF7-LXSN vector control cells grown in rECM for 5–9 days did not exhibit morphologic evidence of apoptosis (Fig. 11 and data not shown). These data suggest that expression of RARβ2 in breast cancer cells grown in contact with rECM may promote the induction of apoptosis.

RETINOIDS AND RETINOIC ACID RECEPTORS REGULATE EXPRESSION OF CBP/P300 IN NORMAL AND MALIGNANT MAMMARY EPITHELIAL CELLS

CBP and p300 are thought to be present in the cell in limiting quantities and it is hypothesized that various transcription factors compete for their binding (Tanaka et al., 1997). Although CBP and p300 are highly homologous and appear to have many overlapping functions, some of their functions are clearly distinct. In F9 cells treated with retinoic acid, both CBP and p300 are required for G_1 arrest and apoptosis but only p300 seems to be required for differentiation (Kawasaki et al., 1998). Furthermore, in regulating growth arrest CBP and p300 appear to target different genes. Finally, mice that are heterozygous nulls for either CBP or p300 have different phenotypes (Tanaka et al., 1997). Transcriptional regulation of CBP or p300 levels has not been demonstrated. However, it was recently shown that HSC-3 oral carcinoma cells treated with 9-cis-retinoic acid exhibit increased levels of CBP and p300 protein on Western blotting (Hayashi et al., 2000, 2001). It has been clearly established that the levels of CBP and p300 expression are very important in regulating gene expression and that alterations in CBP levels have important implications in human disease
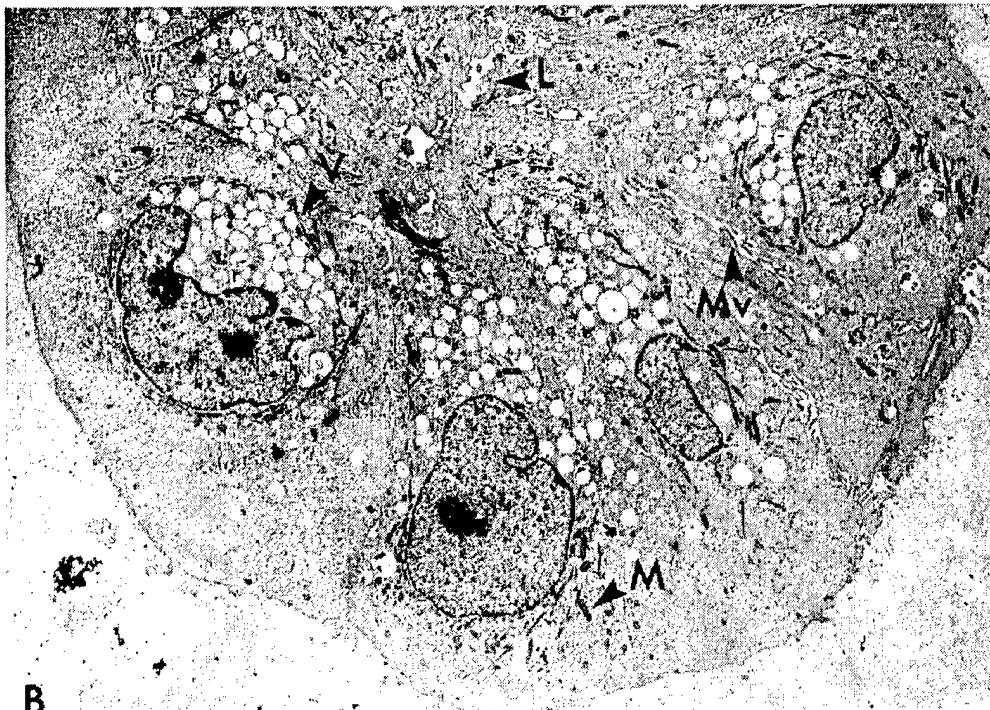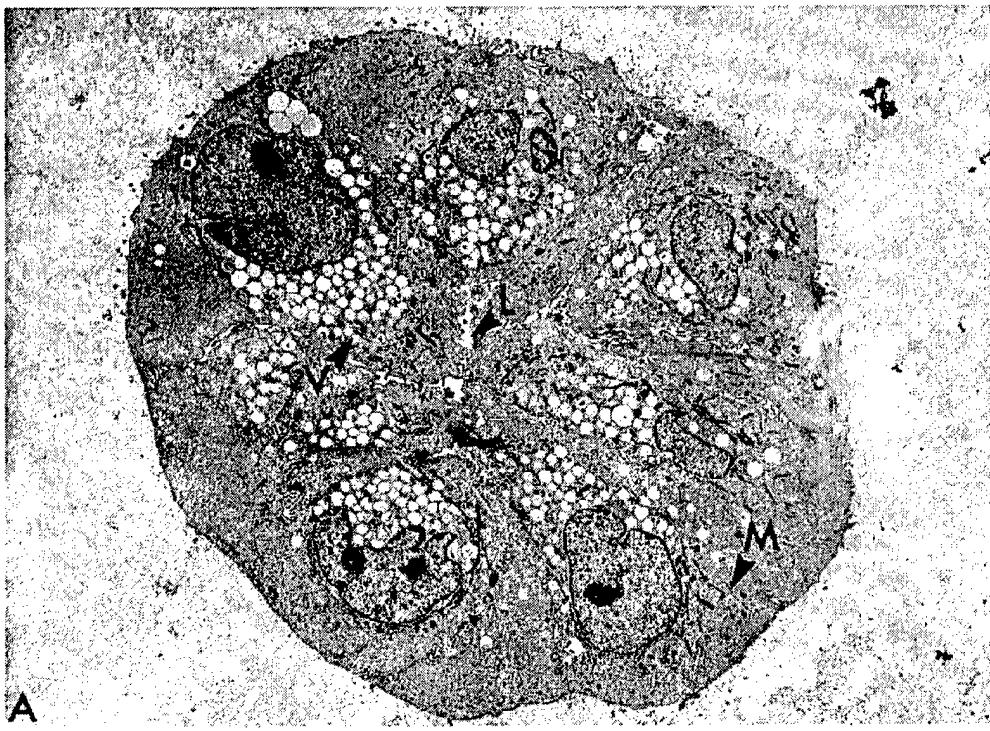
Fig. 9. Phenotypic changes observed by electron microscopy in HMECs expressing a DNRAR suggest that RARs play an important role in the formation of a polarized ductal epithelium. A,B: Electron micrographs of HMEC-LXSN vector control cells grown in rECM for 14 days (magnification of A and B are ×1,500 and ×2,500, respectively). HMEC-LXSN control cells formed acini-like structures that demonstrated a central lumen (L) surrounded by correctly polarized luminal cells consistent with differentiated mammary glandular epithelium. Morphologic evidence of polarity included: 1) microvilli (Mv) were primarily distributed on the luminal and lateral surfaces, 2) secretory vacuoles (V) were present on the luminal surface and not the basal surface, and 3) mitochondria (M) were primarily located at the basal surface. C,D: HMEC-DNRAR transduced cells whose RAR function was inhibited by a DNRAR grown in rECM (magnification C and D are ×1,000 and ×2,500, respectively). Cells grew in large, disorganized multilayered, irregularly shaped colonies, with no lumen formation and a loss of normal cellular polarity. Evidence of lack of polarity included: 1) cells were not organized into spherical, single layer structures, 2) a lumen was not present, 3) mitochondria (M) were present throughout the cell, and 4) secretory vacuoles (V) were randomly distributed throughout the cytoplasm. These morphologic changes observed in HMEC-DNRAR cells suggest that loss of RAR function in HMECs inhibits the formation of a polarized ductal epithelium in vitro.

Figure 9. (Continued.)

Fig. 11. Phenotypic changes observed by electron microscopy in RARβ2-transduced MCF-7 breast cancer cells suggest that RARs play a role in rECM-mediated apoptosis. A: Electron micrographs of MCF7-LXSN vector control cells grown in rECM for 5 days. MCF7-LXSN control cells formed disorganized aggregates of cells. B: In contrast, MCF7-RARβ2 transduced cells grown in rECM exhibited morphologic evidence of apoptosis. Evidence of apoptosis included: 1) cell shrinkage and separation, 2) margination of chromatin (mr), and 3) the presence of apoptotic bodies (ap) containing cytoplasmic elements. Magnification = ×1,500.

A.

HMEC

LXSN   **DNRAR**

-ATRA +ATRA   -ATRA +ATRA

CBP

p300

loading

B.

MCF-7

LXSN   **RARβ2**

-ATRA +ATRA   -ATRA +ATRA

CBP

p300

loading
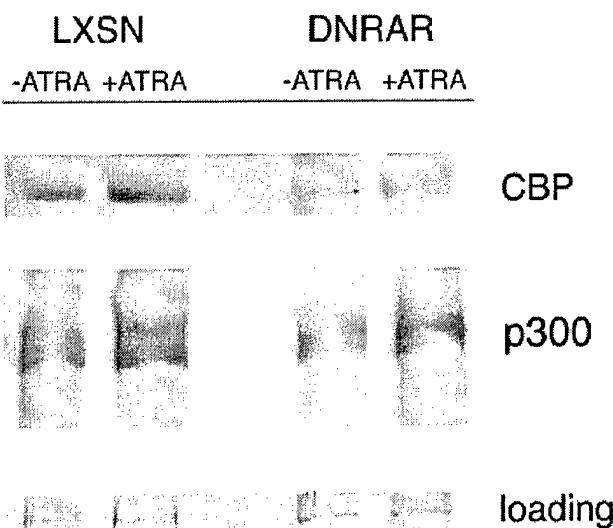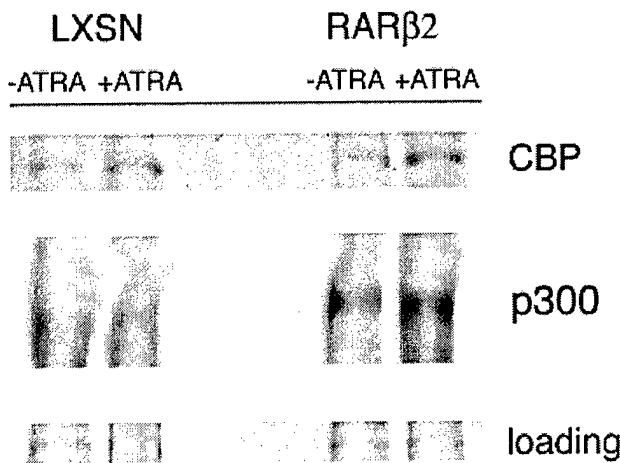
Fig. 12. ATRA-treatment and RAR expression correlate with CBP/p300 expression in normal and malignant mammary epithelial cells. **A:** Western analysis of CBP/p300 protein expression in HMEC-DNRAR cells (DNRAR) and in HMEC-LXSN vector control cells (LXSN). **B:** Expression of CBP/p300 protein in MCF7-RARβ2 transduced breast cancer cell lines (RARβ2) and vector controls (LXSN). Cells were treated with or without 1.0 μM ATRA for 48 hours. The blot was probed with a CBP- and p300-specific polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. An unidentified 90 kd protein band was used as a loading control.

with ATRA or grown in contact with rECM. This is consistent with other reports of differentiation agents mediating growth arrest in normal cells and apoptosis in cancer cells.

Tissue homeostasis is maintained by a dynamic equilibrium between cellular proliferation and cell death (Evans et al., 1998). Apoptosis is felt to be the predominant mechanism of cell death and plays a central role in controlling cell number and in eliminating cells sustaining DNA damage (Thompson et al., 1995). Cancer cells

typically have an immature phenotype representing a block in the normal differentiation pathway. Recently, agents such as retinoids that are capable of inducing differentiation have been utilized in vitro and in vivo in an attempt to restore the normal differentiation program in malignant cells. Restoration of normal growth arrest and differentiation signals in cancer cells appears to activate apoptosis. For example, retinoids induce growth arrest and differentiation of acute promyelocytic leuke-

TABLE 2. *CBP/p300 expression in ATRA-treated transduced normal and malignant mammary epithelial cells*

	HMEC-LXSN		HMEC-DNRAR	
	− ATRA	+ ATRA	− ATRA	+ ATRA
CBP	0.63 ± 0.10	1.0 ± 0.01	0.31 ± 0.07	0.28 ± 0.07
p300	0.88 ± 0.09	1.0 ± 0.01	0.22 ± 0.04	1.16 ± 0.01
	MCF7-LXSN		MCF7-RARβ2	
	− ATRA	+ ATRA	− ATRA	+ ATRA
CBP	0.45 ± 0.06	0.55 ± 0.09	0.44 ± 0.08	1.0 ± 0.01
p300	0.24 ± 0.04	0.24 ± 0.03	0.38 ± 0.04	1.0 ± 0.02

Adherent HMEC-LXSN vector controls, HMEC-DNRAR transduced cells, MCF7-LXSN vector controls, and MCF7-RARβ2 transduced cells were treated for 48 hours with 1.0 μM ATRA. The relative levels of CBP/p300 protein levels were determined by Western analysis and quantitated using Kodak 1D Image Analysis Software. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

mic cells followed by apoptosis (Oshahi et al., 1992; Park et al., 1994) and treatment of human colon adenocarcinoma cells with Herbimycin A results in growth arrest and differentiation followed by apoptosis (Mancini et al., 1997). We observe that ATRA and RARs induce growth arrest followed by apoptosis in RARβ-transduced breast cancer cells, but ATRA induces growth arrest without apoptosis in normal mammary cells. These observations suggest that RAR-initiated normal growth and differentiation signals may promote apoptosis in abnormal cells, perhaps as part of the normal cellular regulatory pathways that target the elimination of damaged cells. We hypothesize that loss of these RAR-derived signals could promote the development of malignancy by permitting the clonal expansion of mutated cells.

CBP and p300 are highly homologous nuclear proteins important for growth regulation and apoptosis. CBP/p300 are known to modulate a diverse range of signaling pathways, including those regulated by estrogens, retinoids, interferon, and p53 (Goldman et al., 1997; Snowden et al., 1998; Vo et al., 2001). Transcriptional regulation of CBP or p300, however, has not been well studied to date and the promoter sequences of CBP and p300 are unknown. There is evidence that RARβ might be critical for CBP/p300 expression (Hayashi et al., 2001). In this study, we observed that 1.0 μM ATRA induced CBP/p300 protein expression in HMECs and in MCF7-RARβ2 cells. Retinoid-resistant MCF7-LXSN cells, lacking RARβ2 expression, treated with 1.0 μM ATRA exhibited no induction of p300 protein expression and only slight increase in CBP protein expression. Suppression of RAR function in HMECs correlated with suppression of CBP/p300 expression. The dominant-negative activity of the DNRAR, RARα403, is sufficient to block induction of CBP but not p300 by 1.0 μM ATRA. Taken together, these results suggest a potential role for ATRA and RARs in mediating the expression of CBP/p300; however, the molecular mechanism for this relationship has not been defined. Since CBP and p300 pools are limited, any alteration in their levels may impact the transcription of genes regulated in part by CBP/p300. It is also important to consider that since the expression of the DNRAR in HMECs increased levels of p300, it is possible that the increased proliferation observed in HMEC-DNRAR cells may reflect increased levels of p300.

Our observations suggest the following model: loss of RARβ2 function in mammary epithelial cells may re-

sult in altered CBP/p300 expression, thereby resulting in loss of growth regulation and promotion of mammary carcinogenesis. Since CBP/p300 are integrators of retinoid signaling and we observe that CBP/p300 protein levels are regulated by RARs and ATRA, it is possible that this loss of growth regulation involves a positive feedback loop. Alternatively, increased expression of a DNRAR such as RARβ4 may result in dysregulated proliferation and promote the clonal expansion of mammary epithelial cells harboring reduced CBP/p300 levels. CBP/p300 are critical regulators of growth arrest, differentiation, and apoptosis. Therefore, loss of RAR function might disrupt this normal cycle of breast epithelial cell homeostasis, leading to an expanded abnormal epithelial cell population. Further mutations of cellular oncogenes or tumor suppressor genes in this expanded population of cells might then lead to the development of overt breast cancer. This model predicts that the loss of RARβ and subsequent downregulation of CBP/p300 might be a relatively early event in the pathogenesis of breast cancer.

NOTE ADDED IN PROOF

Fan et al. (Fan, S, et al. 2002. p300 modulates the BRCA1 inhibition of estrogen receptor activity. Cancer Research 62; 141–151.) have recently shown that p300, but not CBP, transcription and protein levels are downregulated in breast and prostate cancer cell lines by BRCA1. Downregulation of p300 results in the inhibition of estrogen receptor-alpha transcriptional activity.

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Article

Suppression of p53 function in normal human mammary epithelial cells increases sensitivity to extracellular matrix–induced apoptosis

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L ittle is known about the fate of normal human mammary epithelial cells (HMECs) that lose p53 function in the context of extracellular matrix (ECM)–derived growth and polarity signals. Retrovirally mediated expression of human papillomavirus type 16 (HPV-16) E6 and antisense oligodeoxynucleotides (ODNs) were used to suppress p53 function in HMECs as a model of early breast cancer. p53+ HMEC vector controls grew exponentially in reconstituted ECM (rECM) until day 6 and then underwent growth arrest on day 7. Ultrastructural examination of day 7 vector controls revealed acinus-like structures characteristic of normal mammary epithelium. In contrast, early passage p53− HMEC cells proliferated in rECM until day 6 but then underwent apoptosis on day 7. p53− HMEC-E6 passaged in non-rECM culture rapidly (8–10 passages), lost sensitivity to both rECM-induced growth arrest and polarity, and also developed resistance to rECM-induced apoptosis. Resistance was associated with altered expression of α3-integrin. Treatment of early passage p53− HMEC-E6 cells with either α3- or β1-integrin function-blocking antibodies inhibited rECM-mediated growth arrest and induction of apoptosis. Our results indicate that suppression of p53 expression in HMECs by HPV-16 E6 and ODNs may sensitize cells to rECM-induced apoptosis and suggest a role for the α3/β1-heterodimer in mediating apoptosis in HMECs grown in contact with rECM.

Introduction

Interactions between extracellular matrix (ECM)* and mammary epithelial cells are critical for the regulation of cellular proliferation, polarity, and apoptosis (Petersen et al., 1992; Strange et al., 1992). These normal interactions may be disrupted during breast carcinogenesis (Petersen et al., 1992; Zutter et al., 1995). It has been observed that although normal mammary epithelial cells undergo growth arrest in culture in the presence of reconstituted ECM (rECM), breast cancer cells and established breast cancer cell lines fail to ex-

hibit similar growth arrest (Petersen et al., 1992). Thus, it is hypothesized that interaction with ECM may serve to distinguish the growth patterns of normal and malignant mammary epithelial cells.

The mechanism by which ECM-mediated signal transduction events might result in changes in gene expression is a subject of current investigation. It has been shown that rECM regulates both biomechanical and biochemical signaling events and conversely that alterations in cell morphology can alter the response of cells to rECM (Folkman and Moscona, 1978; Roskelley et al., 1994). It is hypothesized that because malignant cells have an altered response to rECM, ECM signaling pathways may utilize tumor suppressor checkpoints critical for cellular organization and polarity (Petersen et al., 1992; Howlett et al., 1994; Weaver et al., 1997).

Integrins are heterodimeric cell surface receptors that link ECM to structural and functional components within the cell (Hynes, 1992). There is increasing evidence that integrins are important regulators of normal mammary mor-
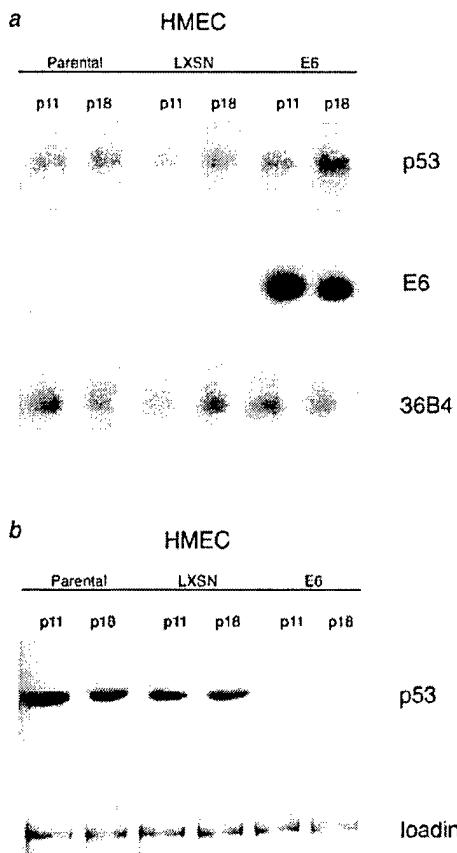
Figure 1. **Expression of p53 and HPV-16 E6 mRNA and p53 proteins in HMECs.** (a) Expression of endogenous p53 and exogenous HPV-16 E6 mRNA in HMECs. Passage 10 and 18 p53+ HMEC-P parental cells (Parental), p53+ HMEC-LXSN controls (LXSN), and p53− HMEC-E6 cells (E6) were analyzed for p53 and HPV-16 E6 mRNA expression. 10 μg of RNA were loaded per lane. 36B4 served as a loading control. (b) Expression of p53 protein is suppressed in p53− HMEC-E6 cells. Passage 10 and 18 p53+ HMEC-P parental cells (Parental), p53+ HMEC-LXSN controls (LXSN), and p53− HMEC-E6 cells (E6) were analyzed for p53 protein expression as described in Materials and methods. Equal amounts of protein lysate were loaded per lane. The protein gel was stained with Coomassie blue, and an unknown 45-kD protein band was used as a loading control.

Figure 2. **Morphologic appearance of early and late passage HMECs expressing HPV-16 E6.** Electron micrographs of early passage (passage 10) p53+ HMEC-LXSN controls (a) and p53− HMEC-E6 cells (b) grown in tissue culture for 4 d were similar in appearance. (c) Late passage (passage 21) p53+ HMEC-LXSN vector control cells were approaching in vitro senescence, were significantly larger than early passage cells, and contained increased numbers of vesicles. (d) Passage 18 p53− HMEC-E6 cells were similar in appearance (but not identical) to passage 10 cells.

phology, since mammary carcinoma cells frequently demonstrate atypical patterns of integrin expression including loss, downregulation, or improper localization (Koukoulis et al., 1991; Glukhova et al., 1995; Lichtner et al., 1998). In the normal mammary gland, the α3/β1 integrin is expressed at the basal surface of luminal epithelial cells (Koukoulis et al., 1991; Glukhova et al., 1995). In contrast, invasive breast carcinomas demonstrate weak staining and redistribution of the α3/β1 integrin. Recently, the α3/β1 integrin has attracted considerable interest, since its function appears to be versatile. For example, the integrin α3/β1 functions as a cell adhesion receptor for laminin-5 (epiligrin), a major ECM protein present in basement membrane (Xia et al., 1996). The α3/β1-integrin is recruited to focal adhesion contacts in cultured cells and thereby plays an important role in linking ECM to components of the actin cytoskeleton (Carter et al.,

1990b; Grenz et al., 1993; DiPersio et al., 1995). Integrin α3/β1 is a critical mediator of intracellular adhesion (Kawano et al., 2001). Studies in keratinocytes suggest that α3/β1 plays a critical role in cell spreading and migration and promotes gap junctional communication (Carter et al., 1990b; Xia et al., 1996; DiPersio et al., 1997). Recently, α3/β1-integrin has been shown to be involved in the initiation apoptosis (Sato et al., 1999). Taken together, these studies illustrate the multifaceted role of α3/β1-integrin in mediating interactions between ECM and epithelial cells and perhaps in initiating apoptosis.

Tissue homeostasis is maintained by a dynamic equilibrium between cellular proliferation and cell death (Evan and Littlewood, 1998). Apoptosis is considered to be the predominant mechanism of cell death and plays a central role in controlling cell number and eliminating cells sustaining DNA damage (Ashkenazi and Dixit, 1998). The role of the tumor suppressor p53 in ECM-induced growth arrest, polarity, and apoptosis is unknown. *TP53* is a cell cycle "checkpoint" gene critical for cell cycle regulation, and it is functionally inactivated in human cancer at a high frequency (Hansen and Oren, 1997). Mutations of the *TP53* gene are detected commonly in breast cancers and are associated with an increased risk of malignancy (Ashkenazi and Dixit, 1998; Levesque et al., 1998). Aberrant expression of p53 in mammary epithelial cells may be a biomarker predicting risk for subsequent breast carcinogenesis. Accumulation of p53 protein in mammary epithelial cells is detected frequently in women at high risk for the development of breast cancer (Fabian et al., 1996) and associated with an increased risk of

progression to breast cancer in women with benign breast disease (Rohan et al., 1998).

Retrovirally mediated introduction of human papillomavirus type 16 (HPV-16) E6 protein into cells provides a model for the isolated loss of p53 function. The E6 protein of the cancer-associated HPV-16 binds to p53 and targets it for degradation through the ubiquitin pathway (Demers et al., 1996). We employed this approach and antisense (AS) oligondeoxyucleotides (ODNs) to acutely suppress p53 function in normal human mammary epithelial cells (HMECs) in order to model p53 loss in the context of ECM signaling.

Our results showed that control HMECs expressing p53 underwent rECM-mediated growth arrest and formed a polarized epithelium. In contrast, HMECs with HPV-16 E6– and ODN-suppressed p53 expression underwent rECM-induced growth arrest followed by apoptosis. p53$^-$ HMEC-E6 cells passaged in non-rECM culture rapidly acquired resistance to rECM-mediated growth arrest, polarity, and apoptosis after 8 –10 passages in culture. Treatment of early passage p53$^-$ HMEC-E6 cells with either α3- or β1-integrin function-altering antibodies (Abs) blocked rECM-mediated growth arrest and induction of apoptosis. Observations in our model system suggest that rECM may play an important role in the induction of apoptosis in early passage p53$^-$ HMECs via an α3/β1 signaling pathway.

Results

p53 protein suppression in HMECs

Retrovirally mediated expression of the HPV-16 E6 protein was used to suppress normal intracellular p53 protein levels in HMECs. The pLXSN16E6 retroviral vector containing the coding sequence for HPV-16 E6 viral protein has been described previously (Demers et al., 1994). Actively dividing passage 9 AG11132 cells were infected with the retroviral vector LXSN16E6 or the control vector LXSN, and the infected cells were selected as described in Materials and methods. All experiments described subsequently were performed on mass cultures. HMEC-P parental cells, p53$^+$ HMEC-LXSN controls, and p53$^-$ HMEC-E6 cells were passaged serially in culture on plastic tissue culture plates. As previously observed (Seewaldt et al., 1999a,b), p53$^+$ HMEC-LXSN controls and HMEC-P parental cells exhibited a progressive increase in doubling time with continued serial passaging and entered "phase b" growth plateau at about passage 23 (Romanov et al., 2001). In contrast, p53$^-$ HMEC-E6 cells exhibited an extended lifespan in culture and entered a growth plateau at passage 40.

Northern blot analysis was performed on p53$^+$ HMEC-P parental cells, p53$^-$ HMEC-E6 cells, and p53$^+$ HMEC-LXSN controls (passage 11 and 18) to test for HPV-16 E6 mRNA expression. Expression of the LTR-initiated E6 mRNA transcript was observed in p53$^-$ HMEC-E6 cells at passage 11 and 18 but not in parental or vector controls (Fig. 1 a).

Western blots were performed on p53$^+$ HMEC-P parental cells, p53$^-$ HMEC-E6 cells, and p53$^+$ HMEC-LXSN controls (passage 11 and 18) to determine the relative levels of p53 protein expression. Expression of p53 protein was observed in p53$^+$ HMEC-P parental cells and p53$^+$ HMEC-
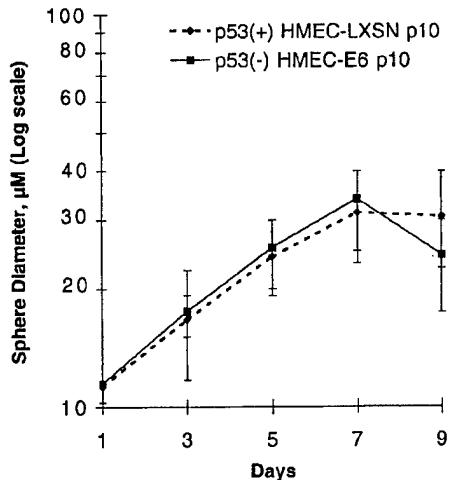
Figure 3.   rECM-induced growth arrest of early passage p53$^-$ HMEC-E6 cells and p53$^-$ HMEC-LXSN controls. The mean diameter of spheres formed by p53$^+$ HMEC-LXSN controls (passage 10) and p53$^-$ HMEC-E6 cells (passage 10) were plotted as a function of days in culture. Cells were plated in rECM on day 0 and the diameter of growing spherical cell colonies measured with an eye piece equipped with micrometer spindle. For both p53$^+$ HMEC-LXSN controls and p53$^-$ HMEC-E6 cells, the 20 largest colonies were measured at each time point. These data are representative of three separate experiments.

LXSN vector controls but was not detectable by Western analysis in p53$^-$ HMEC-E6 cells at either passage 11 or 18 (Fig. 1 b).

Cytogenetic analysis of early and late passage p53$^+$ and p53$^-$ HMECs

Cytogenetic analysis of p53$^+$ HMEC-P parental cells, p53$^+$ HMEC-LXSN controls, and early passage p53$^-$ HMEC-E6 cells was performed using both spectral karyotyping (SKY) and DAPI staining to (a) test whether parental and vector control cells exhibited a normal karyotype and (b) verify that early passage p53$^-$ HMEC-E6 cells did not exhibit specific karyotypic abnormalities.

38 early passage p53$^+$ HMEC-P parental cells (passage 8) were karyotyped. 35 (92%) metaphase cells had a normal diploid karyotype. Three cells (8%) exhibited random chromosome loss.

Similarly, 22 early passage p53$^+$ HMEC-LXSN control cells (passage 10) were karyotyped. 20 (91%) p53$^+$ HMEC-LXSN metaphase cells had a normal diploid karyotype. Two cells (9%) exhibited random chromosome loss.

22 late passage p53$^+$ HMEC-LXSN control cells (passage 17) were karyotyped. 18 (82%) p53$^+$ HMEC-LXSN metaphase cells had a normal diploid karyotype. Three cells (13%) exhibited random chromosome loss. One cell (5%) was tetraploid (92 chromosomes). These results are consistent with karyotypes reported for late "phase a" (pregrowth plateau) HMECs (Romanov et al., 2001).

A total of 21 early passage p53$^-$ HMEC-E6 metaphase cells (passage 10) were karyotyped. Two cells were analyzed by SKY, and 19 cells were analyzed using inverted and contrast-enhanced DAPI staining. The majority of cells (12

Table I. **Karyotype of late passage p53⁻ HMEC-E6 cells**

Cell number	Karyotype
S14	32<2n>,XX,−1,−2,−5,−8,−11,−12,−14,−16,−16,−17,−18,−20,−21, der(10;21)(q10;q10),−22,+mar
S21	35,XX,−2,−3,−5,−6,−7,−8,−11,dic(12;20)(p1?1;p13),−16,del(16)(p11),−18,−19
S24	36,−X,t(X;14)(q2?8;q24),r(5),der(6)del(6)(p22)del(6)(q11),−9,−10,−10,−13, dic(14;21)(q32;q22),−15,−15,der(17;21)(q10;q10),−22
S32	36,XX,−1,−3,−5,−6,del(8)(q13q24),−9,−11,−13,−17,−18,−19
S17	39,X,−X,−1,del(3)(p11),der(6;21)(q10;q10),del(8)(q24),del(12)(p13),−15,del(16)(p11),−18,−19,−21
S19	38,XX,−1,−2,−5,−7,dic(9;20)(p11;p13),−15,dic(16;21)(p11;p13),−18
S6	40,XX,del(1)(q3?1),−3,der(6)t(3;6)(?;p11),−10,der(12;16)(q10;q10),−15,−17,−10, der(?21)t(20;?21)(p11;q11)
S30	41,X,dic(X;12)(q28;p11),del(8)(?p11),der(16;21)(q10;q10),der(15;21)(q10;q10), dic(17;18)(p11;p11.3),−22
S33	41,XX,dic(5;21)(p1?5;p1?3),dic(12;21)(p1?2;q22),dic(15;17)(p13;q25),dic(16;22)(p11;p1?3), dic(18;20)(p11.3;q13.?1)
S13	41,XX,−1,−4,−6,−10,dic(12;17)(p13;p1?3),add(16)(p11),−21
S34	42,XX,del(2)(q11),del(9)(q11),del(12)(p1?2),dic(12;16)(p11;q24),−15,−21,−21
S7	44,XX,dic(6;8)(p25;p23),−13,del(17)(p11),der(21;22)(q10;q10)
S9	44,X,dic(X;21)(p22;p1?1),del(2)(q11),+der(2),+der(2)t(2;16)(?;?q1?3),−16,−17,−19
S2	44,XX,del(7)(q11),trc(7;21;20)(q36;p11q21−22;p13),del(16)(?p11)
S20	44,XX,der(12;16)(q10;q10),dic(20;21)(p13;p1?1)
S26	44,XX,dic(10;17)(q26;p1?1),−12
S28	44,X,dic(X;15)(q11;p11),−16
S29	44,XX,−5,del(16)(p11)
S27	45,XX,−10,der(16)t(10;16)(q11;p11),t(20;20)(q13.1;q13.3)
S16	45,XX,+15,dic(15;15)(q26;p1?3),der(12;16)(q10;q10),der(20)t(18;20)(?q12;p13)
S5	45,XX,dic(14;21)(q3?2;p11),−15,del(16)(p11),del(17)(p11),+21,der(21)t(14;21)(?;?p11)
S18	46,XX,csb(2)(p11),del(9)(q12),del(12)(p13),der(16;21)(q10;q10),dic(20;21)(p13;p13)
S10	49,XX,+2,dic(3;6)(p26;p25)×2,+dic(3;22)(p11;p13),+5,+5,t(5;9)(q35;q34), dic(6;20)(p25;q13.?1),−7,+8,+10,+dic(12;6;19)(p13;?;p13.?3),−13,+14,−15,+16, der(16)t(13;16)(q1?2;p13)x2,+der(17)t(17;19)(p11;?p12),+18,dic(20;20)(p13;q13.3),−21
S15	55,X,t(X8)(q2?8p21),+der(X)t(X2)(p22?),−1,del(2)(q11),+del(2)(?p11),+3,+4,+5,+5,+7,+7,−9,+11, +11,dic(12;21)(p13;q22)?dup(12)(p11p13),+13,+14,−16,del(16)(p1?2),−19,+20,+22
S35	61<3n>,−X,−X,−X,−1,−2,−3,−3,−4,+5,−7,−7,+8,+9,−12, der(12)t(X;12)(q21;p13),+14,−15,−16,−20
S1	63<3n>,XXX,−2,−3,der(3)del(3)(p21)del(3)(q2?3),+4,der(6)inv(6)(p25q23)dic(66)(p25q27),+7,+10, +11,+12,dic(12;22)(p13;p13)×2,−14,−15,−16,−16,dic(20;20)(p13;q13.3),−21,der(21;21)(q10;q10)
S25	84,XXX,del(X)(q13),−3,−5,del(6)(p11),−8,−8,dic(14;16)(p11;p11)×2,dic(17;21)(p13;q22)×2
D1	46,XX,del(16)(p1?1)
D3	43,XX,−15,−19,−20
D10	41,X,−X,dic(7;21)(p22;q22),−11,dic(12;?)(p13;?),−15,dic(16;?)(p1?1;?),−20,−21,−22,+2mar
D6	44,XX,−6,der(12;16)(q10;q10)
D9	44,XX,der(16;20)(q10;q10),der(17;21)(q10;q10)
D12	41,XX,−12,add(15)(p11−13),−16,add(17)(p1?1),−20,−21,−21
D14	44,XX,+9,dic(?9;12)(q34;p13),der(16;17)(q10;q10),dic(21;21)(q22;q22)
D2	45,XX,der(12;14)(q10;q10)

cells, 57%) had a normal diploid chromosome content, three cells had random chromosome loss (14%), and the remaining cells were aneuploid. In three cells (14%), multiple losses of whole chromosomes occurred, resulting in the chromosomes number of 30, 32, and 36, respectively. The other three cells (14%) were either tetraploid (92 chromosomes) or hypotetraploid (90 and 80 chromosomes). Only two cells, both near diploid and both studied using DAPI staining, displayed structural chromosome changes: inv(20)(p11q13.1) in one cell, and del(X)(p21), dic(14; 19)(q32;q13.4), and a marker chromosome in another.

In contrast to early passage cells, late passage p53⁻ HMEC-E6 (passage 18) were markedly abnormal with numerical and structural chromosome aberrations. A total of 35 metaphase cells were analyzed: 27 using SKY and 8 using inverted and contrast-enhanced DAPI staining (Table I).

These results have been published previously (Seewaldt et al., 2001). No cell had a normal diploid karyotype. The predominant types of structural changes were deletions, whole arm translocations, and dicentric chromosomes with breakpoints in the pericentromeric and/or telomeric regions. Although a majority of the 35 cells contained complex chromosomal rearrangements, each resistant cell analyzed was unique. This suggests that chromosome aberrations observed in the late passage p53⁻ HMEC-E6 cell population resulted from a generalized event causing karyotypic instability that is inconsistent with the outgrowth of a mutant clone.

Morphologic appearance of early and late passage HMECs with HPV-16 E6–suppressed p53 expression

Early passage p53⁺ HMEC-LXSN controls (passage 10) and p53⁻ HMEC-E6 cells (passage 10) were similar in appear-
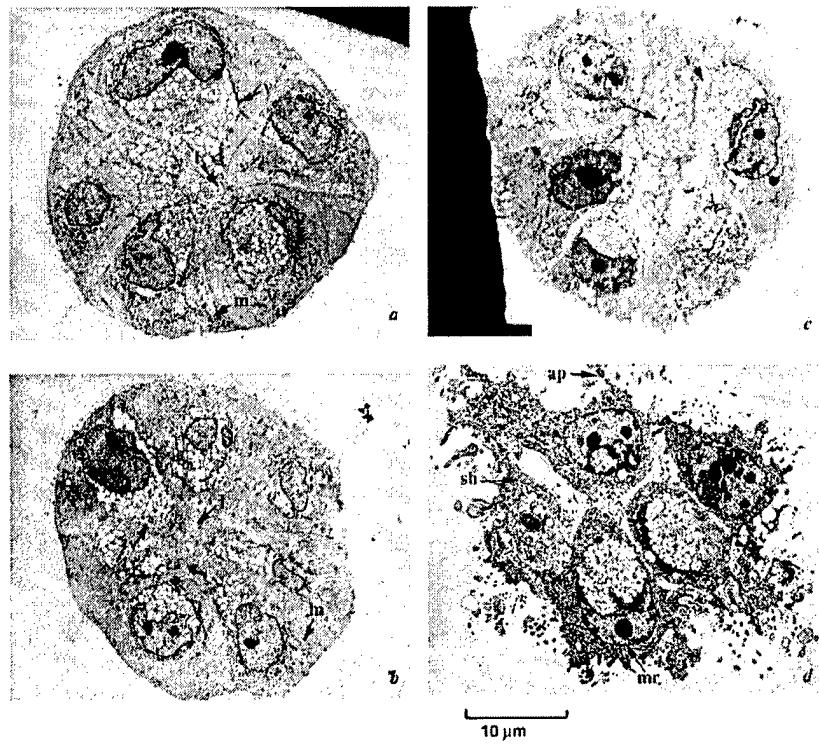
Figure 4. **Early passage p53⁻ HMEC-E6 cells undergo apoptosis when cultured in contact with rECM starting at 7 d.** (a and b) Electron micrographs of p53⁺ HMEC-LXSN vector control cells (passage 10) grown in rECM for 7 (a) and 14 (b) d. p53⁺ HMEC-LXSN controls formed acini-like structures, which demonstrated a central lumen (l) consistent with nonlactating mammary glandular epithelium: (1) nuclei were aligned predominantly with the basal surface, (2) secretory vesicles (v) were present on the luminal surface and not on the basal surface, and (3) mitochondria (m) were located at the basolateral surface. (c and d) p53⁻ HMEC-E6 cells grown in rECM for 6 (c) and 7 (d) d. At 6 (c), p53⁻ HMEC-E6 cells are organized around a central lumen, there is cell separation (s), nuclei are primarily oriented at the basal surface, and vesicles (v) are present typically at the luminal surface. At 7 d (d), p53⁻ HMEC-E6 cells demonstrated evidence of apoptosis including (1) nuclear condensation, (2) cell shrinkage (sh) and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements.

ance (Fig. 2, a and b). Late passage p53⁺ HMEC-LXSN cells (passage 21) exhibited morphologic changes characteristic of senescence including increased cell size and increased cytoplasmic vesicles (Fig. 2 c). In contrast, late passage p53⁻ HMEC-E6 cells (passage 18) were morphologically similar (but not identical) to passage 10 cells (Fig. 2 d).

rECM-induced growth arrest of HMECs is not altered by the acute expression of HPV-16 E6

Early passage p53⁺ HMEC-LXSN controls (passage 10) and p53⁻ HMEC-E6 cells (passage 10) were grown in prepared rECM as a single cell suspension. Both p53⁺ HMEC-LXSN and p53⁻ HMEC-E6 cells grew exponentially in rECM until day 6 and then growth arrested on day 6–7 (Fig. 3). They formed a uniform population of spherical colonies. The mean diameter of p53⁺ HMEC-LXSN and p53⁻ HMEC colonies on day 7 was 30.5 ± 7.4 and 33 ±5.5 μm, respectively (Fig. 3). There was a decrease in the mean sphere diameter of p53⁻ HMEC-E6 cells relative to controls on day 9, but this was not statistically significant. These data suggest that the acute suppression of p53 function in HMECs by HPV-16 E6 alters neither proliferation of cells in contact with rECM nor rECM-induced growth inhibition as measured by colony diameter in this in vitro system.

Acute expression of HPV-16 E6 induces apoptosis in rECM-growth arrested HMECs in the absence of p53 expression

EM was performed on early passage rECM growth-arrested p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells to test whether suppression of p53 expression by HPV-16 E6 altered the response of HMECs to rECM. p53⁺ HMEC-

LXSN control cells (passage 10) proliferated in contact with rECM until day 6 and then underwent rECM-induced growth arrest on day 6–7.

As observed previously (Seewaldt et al., 1997b), day 7 p53⁺ HMEC-LXSN cells grown in rECM exhibited an acinus-like structure consistent with normal nonlactating mammary glandular epithelium (Fig. 4 a). There was no evidence of apoptosis. Day 14 p53⁺ HMEC-LXSN cells likewise did not exhibit evidence of apoptosis (Fig. 4 b).

In contrast, early passage p53⁻ HMEC-E6 cells (passage 10) proliferated in rECM until day 6 and then underwent
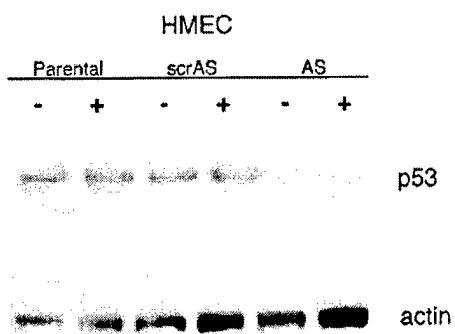
Figure 5. **Expression of p53 protein is suppressed in p53⁻ HMEC-AS cells.** Passage 10 p53⁺ HMEC-P parental cells (Parental), p53⁺ HMEC-scrAS controls (scrAS), and p53⁻ HMEC-AS cells (AS) were grown with (+) or without rECM (−) and analyzed for p53 protein expression as described in Materials and methods. Equal amounts of protein lysate were loaded per lane. Hybridization with Abs to actin serves as the loading control.
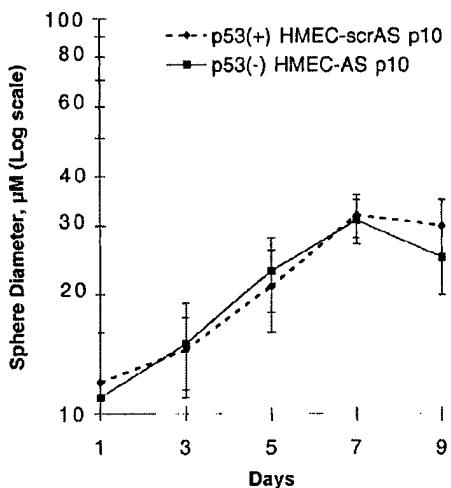
Figure 6. rECM-induced growth arrest of early passage p53⁻ HMEC-AS cells and p53⁺ HMEC-scrAS controls. The mean diameter of spheres formed by p53⁺ HMEC-scrAS controls (passage 10) and p53⁻ HMEC-AS cells (passage 10) are plotted as a function of days in culture. Cells were plated in rECM on day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with micrometer spindle. The 20 largest colonies were measured at each time point. These data are representative of three separate experiments.

apoptosis on day 7. There was no morphologic evidence of apoptosis in day 6 rECM cultures (Fig. 4 c). On day 7, 100 early passage p53⁻ HMEC-E6 cell clusters were surveyed for the presence or absence of apoptosis. 98 clusters (98%) exhibited morphologic evidence of apoptosis by the following criteria: (a) nuclear condensation, (b) cell shrinkage and separation, (c) margination of chromatin, (d) the presence of apoptotic bodies, and (e) mitochondrial condensation (Fig. 4 d).

A second HMEC strain, AG11134, was tested to ensure that these observations were not HMEC strain specific. Similar to observations made in HMEC strain AG11132 above, (a) AG1134-LXSN controls underwent growth arrest and formed an acinus-like structure in contact with rECM at day 7 (unpublished data), and (b) early passage AG11134-E6 cells exhibited morphologic evidence of apoptosis at day 7 (unpublished data).

The terminal deoxynucleotidyl transferase (TdT) method was also used to detect the presence or absence of apoptotic strand breaks in day 7 rECM culture. The 3′-hydroxyl termini of apoptotic-induced strand breaks were labeled with biotin-dUTP by exogenous TdT and were detected in situ by HRP-conjugated streptavidin. Day 7 rECM growth-arrested early passage p53⁺ HMEC-LXSN controls (passage 10) did not demonstrate evidence of apoptosis (unpublished data). In contrast, early passage day 7 p53⁻ HMEC-E6–transduced cells (passage 10) grown in contact with rECM exhibited apoptotic strand breaks (unpublished data).

These observations indicate that whereas early passage p53⁺ HMEC-LXSN controls undergo rECM-induced growth arrest, early passage p53⁻ HMEC-E6 cells undergo rECM-mediated growth arrest on day 6 followed by induction of apoptosis on day 7. Results from this in vitro model of rECM-induced apoptosis in HMECs suggest an association between HPV-16 E6–induced suppression of p53 function and the induction of rECM-mediated apoptosis.
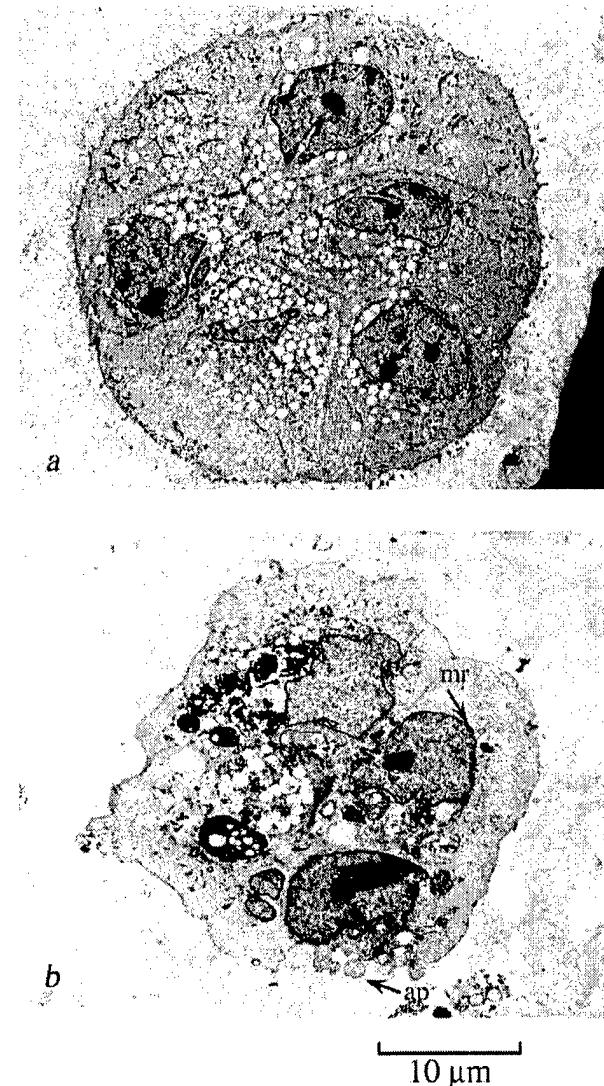
Figure 7. Early passage p53⁻ HMEC-AS cells undergo apoptosis when cultured in contact with rECM starting at 7 d. (a) Electron micrographs of p53⁺ HMEC-scrAS control cells (passage 10) grown in rECM for 14 d. p53⁺ HMEC-LXSN controls formed acini-like structures. (b) p53⁻ HMEC-AS cells grown in rECM for 7 d demonstrated evidence of apoptosis including (1) nuclear condensation, (2) cell shrinkage and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements.

Acute suppression of p53 by an AS approach in HMECs promotes sensitivity to rECM-mediated apoptosis

Since HPV-16 E6 may have effects other than the suppression of p53, an AS approach was used to test whether the acute suppression of p53 function promotes sensitivity to rECM-mediated apoptosis. p53 protein expression was suppressed using a p53 AS ODN in HMECs. Western blot analysis demonstrated almost complete suppression of p53 protein expression in HMECs treated with the p53 AS ODN (p53⁻ HMEC-AS) and no suppression of p53 protein expression in HMECs treated with a scrambled sequence of the p53 AS ODN (p53⁺ HMEC–scrambled AS [scrAS]) (Fig. 5).
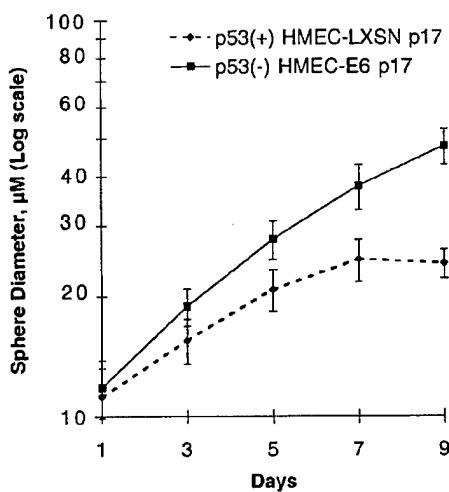
Figure 8. **Late passage p53⁻ HMEC-E6 cells are resistant to rECM-induced growth arrest.** The mean diameter of spheres formed by late passage p53⁺ HMEC-LXSN controls (passage 17) and p53⁻ HMEC-E6 cells (passage 18) are plotted as a function of days in culture as described in Materials and methods. These data are representative of three separate experiments.

Consistent with results obtained in early passage p53⁻ HMEC-E6 cells, the acute suppression of p53 function by an AS approach did not alter the ability of p53⁻ HMEC-AS or p53⁺ HMEC-scrAS controls to proliferate in rECM or to undergo rECM-mediated growth arrest (Fig. 6). Mean diameter of p53⁺ HMEC-scrAS and p53⁻ HMEC-AS colonies on day 7 was 32 ± 4.0 and 31 ± 5.0 μm, respectively (Fig. 6). These data suggest that the acute suppression of p53 function in HMECs by an AS approach neither alters proliferation of cells in contact with rECM nor does it alter rECM-induced growth inhibition as measured by colony diameter.

We tested whether inhibition of p53 protein expression by an AS approach promoted rECM-mediated apoptosis. On day 7, 100 early passage p53⁻ HMEC-AS cell clusters were surveyed for the presence or absence of apoptosis. Similar to early passage p53⁻ HMEC-E6 cells, p53⁻ HMEC-AS cells underwent apoptosis on day 7 (Fig. 7 b). 90 clusters (90%) exhibited morphologic evidence of apoptosis by the following criteria: (a) nuclear condensation, (b) cell shrinkage and separation, (c) margination of chromatin, (d) the presence of apoptotic bodies, and (e) mitochondrial condensation (Fig. 7 b). In contrast, early passage p53⁺ HMEC-scrAS cells did not exhibit morphologic evidence of apoptosis on day 7 or 14 (Fig. 7 a; unpublished data). These observations support the hypothesis that the acute suppression of p53 function promotes rECM-mediated apoptosis.

Late passage p53⁻ HMEC-E6 cells acquire resistance to rECM-induced growth arrest

Starting at passage 16–18, p53⁻ HMEC-E6 cells acquired resistance to rECM-induced growth arrest as evidenced by continued increase in sphere diameter from day 5 to 9 (Fig. 8). The mean sphere diameter was 37 ± 3 μm at day 7 and 48 ± 5 μm at day 9. In contrast, passage 17 p53⁺ HMEC-LXSN cells exhibited sensitivity to rECM-induced growth arrest (Fig. 8).

Late passage HMECs with HPV-16 E6-suppressed p53 function exhibit dysregulated growth and are resistant to rECM-induced apoptosis

Late passage p53⁺ HMEC-LXSN controls (passage 17) and p53⁻ HMEC-E6 cells (passage 18) were cultured in rECM. In contrast to early passage p53⁻ HMEC-E6 cells, late passage cells demonstrated disorganized growth and no evidence of apoptosis at day 7 or 14 (Fig. 9, a and b). Similar to early passage p53⁺ HMEC-LXSN vector controls, electron micrographs of late passage controls demonstrated a normal acinus-like structure without evidence of apoptosis at day 7 (unpublished data) and 14 (Fig. 9 c). Passage 18 p53⁻ HMEC-E6 cells grown in rECM formed disorganized multilayered irregularly shaped colonies (Fig. 9, a and b). There was no morphologic evidence of apoptosis in passage 18 p53⁻ HMEC-E6 cells grown in rECM. A second HMEC strain AG11134 was tested to confirm that these results were not strain specific. Late passage AG11134-E6 cells were grown in rECM. Similar to late passage p53⁻ HMEC-E6 cells, AG11134-E6 cells formed disorganized aggregates of cells and did not demonstrate evidence of apoptosis (unpublished data). These results suggest that p53⁻ HMEC-E6 cells passaged in vitro acquire resistance to rECM-induced apoptosis.

Late passage p53⁻ HMEC-E6 cells grown in contact with rECM lack polarized expression of E-cadherin

Early and late passage p53⁺ HMEC-LXSN and early passage p53⁻ HMEC-E6 cells grown in contact with rECM demonstrated basolateral expression of E-cadherin, including lateral staining at cell–cell junctions consistent with a polarized epithelium (Fig. 10, a–c). 20 cell clusters were surveyed per data point. These results are similar to what is observed in normal breast sections and consistent with results obtained by other investigators when mammary epithelial cells are grown in contact with rECM (Weaver et al., 1997; Spancake et al., 1999). In contrast, late passage p53⁻ HMEC-E6 cells exhibited punctate dispersed and intracellular staining consistent with a loss of epithelial polarity (Fig. 10 d). These results are similar to what is observed in tumorigenic mammary epithelial cells that lack epithelial polarity grown in rECM (Weaver et al., 1997).

Laminin is critical for the induction of apoptosis in early passage p53⁻ HMEC-E6 cells

To identify the component of rECM that promotes apoptosis, early passage p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN controls were cultured in growth factor–depleted Matrigel™, collagen I/IV gels, and collagen I/IV supplemented with laminin. Early passage p53⁻ HMEC-E6 cells underwent apoptosis when cultured for 7 d in growth factor–depleted Matrigel™ (Fig. 11 a), suggesting that depletion of growth factors present in the Matrigel™ are not critical for the induction of apoptosis. Early passage p53⁺ HMEC-LXSN controls did not undergo apoptosis when cultured in growth factor–depleted Matrigel™ (Fig. 11 b). When early passage p53⁻ HMEC-E6 cells were cultured in the presence of collagen I/IV, there was no evidence of apoptosis after 7 and 14 d (Fig. 11 c; unpublished data). Likewise, early passage p53⁺ HMEC-LXSN controls did not exhibit evidence of apoptosis when grown in the presence of
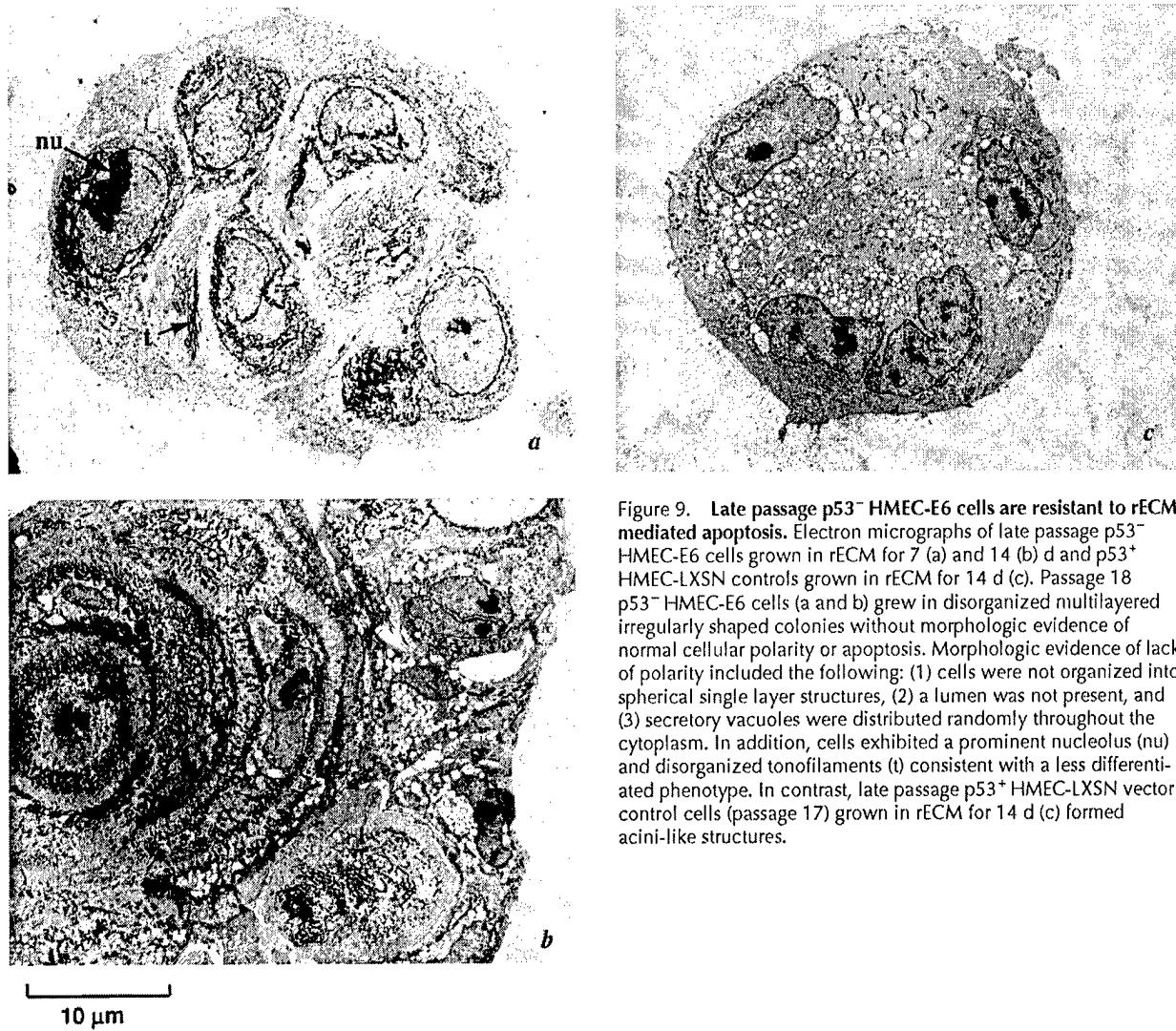
Figure 9. **Late passage p53⁻ HMEC-E6 cells are resistant to rECM-mediated apoptosis.** Electron micrographs of late passage p53⁻ HMEC-E6 cells grown in rECM for 7 (a) and 14 (b) d and p53⁺ HMEC-LXSN controls grown in rECM for 14 d (c). Passage 18 p53⁻ HMEC-E6 cells (a and b) grew in disorganized multilayered irregularly shaped colonies without morphologic evidence of normal cellular polarity or apoptosis. Morphologic evidence of lack of polarity included the following: (1) cells were not organized into spherical single layer structures, (2) a lumen was not present, and (3) secretory vacuoles were distributed randomly throughout the cytoplasm. In addition, cells exhibited a prominent nucleolus (nu) and disorganized tonofilaments (t) consistent with a less differentiated phenotype. In contrast, late passage p53⁺ HMEC-LXSN vector control cells (passage 17) grown in rECM for 14 d (c) formed acini-like structures.

collagen I/IV (Fig. 11 d). Interestingly, neither early passage p53⁺ HMEC-LXSN controls nor p53⁻ HMEC-E6 cells formed acinar structures when grown in collage I/IV (Fig. 11, c and d). When human placental laminin was added to the collagen I/IV gel, both early passage p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN vector controls developed branching tubular structures that terminated in spherical aggregates (Fig. 11, e and g). Early passage p53⁻ HMEC-E6 cells present in both branched and aggregate structures exhibited evidence of apoptosis at 7–10 d (Fig. 11 g). By day 14, no cells were visualized by EM (unpublished data). Likewise, early passage p53⁻ HMEC-E6 cells cultured on a laminin-coated substratum in the presence of a 1:1 dilution of standard medium to laminin formed similar three-dimensional branching aggregates that also underwent apoptosis on days 7–10 as determined by EM (unpublished data). In contrast, early passage p53⁺ HMEC-LXSN controls grown in collagen I/IV supplemented with laminin did not exhibit evidence of apoptosis at 14 d (Fig. 11 h). These observations suggest a critical role for laminin in the induction of apoptosis in p53⁻ HMEC-E6 cells.

α3-integrin expression is altered in rECM-resistant late passage p53⁻ HMEC-E6 cells

Early and late passage p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells were tested for α2-, α3-, α6-, β1-, and β4-integrin expression by immunohistochemistry. All early and late passage p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6 cells stained positively for α2-, α3-, and β1-integrins and very weakly for α6- and β4-integrins (Fig. 12; unpublished data). Late passage p53⁻ HMEC-E6 cells grown in non-rECM culture exhibited a qualitative decrease in expression of α3-integrin (Fig. 12 d).

Early and late passage p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells were grown in rECM and tested for α3- and β1-integrin expression by immunohistochemistry (clones P1F2 and P4C10, respectively). Early and late passage p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6 cells exhibited polarized basal expression of α3- and β1-integrins (Fig. 13). In contrast, late passage p53⁻ HMEC-E6 cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of α3-integrins (Fig. 13 d). Redistribution of α3-integrins has been seen

25 µm
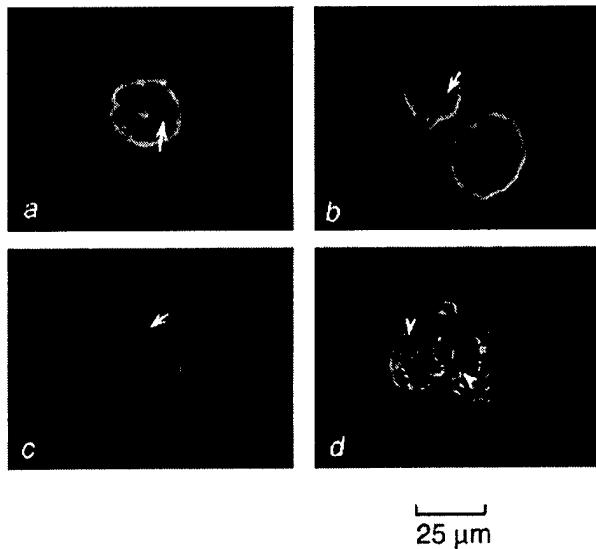
Figure 10. **Localization of E-cadherin in p53[+] HMEC-LXSN and p53[−] HMEC-E6 cells using immunofluorescence microscopy.** Frozen section of early passage p53[+] HMEC-LXSN controls (passage 11) (a), early passage p53[−] HMEC-E6 cells (passage 11) (b), late passage p53[+] HMEC-LXSN controls (passage 16) (c), and late passage p53[−] HMEC-E6 cells (passage 21) (d) grown in rECM for 6 d, cryosectioned, and stained with a mAb to E-cadherin as described in Materials and methods. E-cadherin was localized at the basolateral surface and at points of cell–cell contact in early and late passage p53[+] HMEC-LXSN cells and in early passage p53[−] HMEC-E6 cells (a–c, arrows). In contrast, late passage p53[−] HMEC-E6 cells showed punctate dispersed membrane and intracellular staining (d, arrowheads).

previously by other investigators in association with loss of mammary epithelial cell polarity in rECM culture and is consistent with these findings (Weaver et al., 1997). Late passage p53[−] HMEC-E6 cells grown in rECM exhibited polarized basal β1-integrin expression but had an increase in the amount of cytosolic expression relative to early passage cells (Fig. 13 h).

α3- and β1-integrin function-altering Abs inhibit ECM-induced growth arrest of early passage p53[+] and p53[−] HMEC-E6 cells

Early passage p53[−] HMEC-E6 cells (passage 10) were treated with either α3- or β1-integrin function-altering Abs or control mouse nonimmune IgG and grown in prepared rECM as a single cell suspension. As expected, p53[+] HMEC-LXSN controls and p53[−] HMEC-E6 cells treated with nonimmune mouse IgG or β1-integrin–stimulating Ab (clone B3B11) grew exponentially in rECM until day 6 and then growth arrested on day 6–7 (Fig. 14 a; unpublished data), forming a uniform population of spherical colonies. In contrast, p53[+] HMEC-LXSN controls and p53[−] HMEC-E6 cells treated with α3- or β1-integrin blocking Abs (clones P1B5 and JB1A, respectively) did not undergo growth arrest on day 6–7 and continued to proliferate (Fig. 14, b and c). Mean diameter of control p53[−] HMEC-E6 colonies treated with nonspecific mouse IgG on day 7 and 9 was 30 ± 3 and 27 ± 4 µm, respectively (Fig. 14 a). Mean diameter of p53[−] HMEC-E6 cells treated with α3- or β1-integrin blocking Abs on day 9 was 51 ± 5 and 52 ± 4 µm, respectively (Fig. 14 c). p53[+] HMEC-LXSN controls
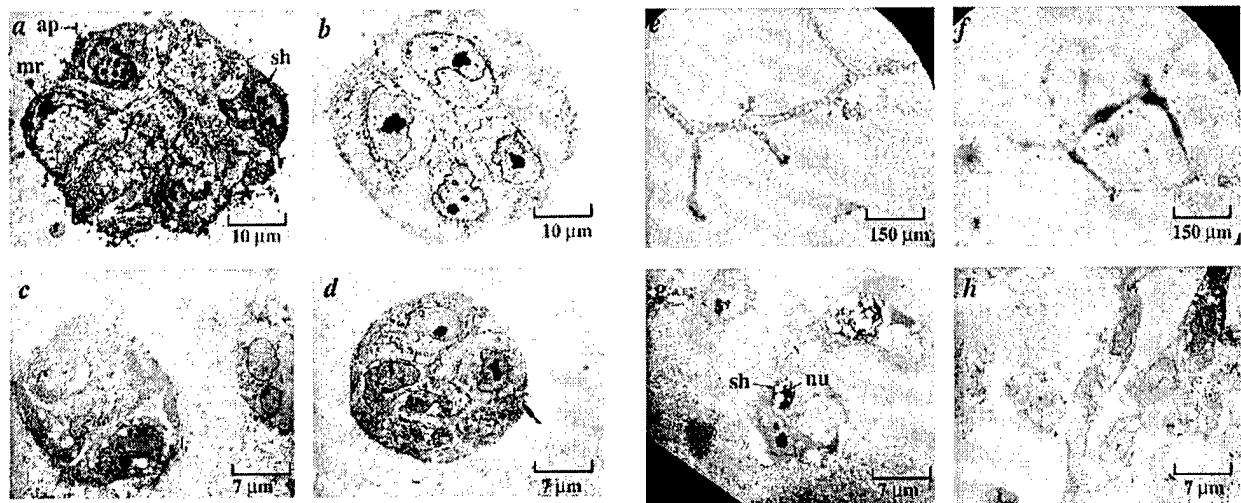
Figure 11. **Early passage p53[−] HMEC-E6 cells undergo apoptosis when cultured in contact with growth factor–depleted rECM and laminin as evidenced by EM.** At 7 d (a), passage 11 p53[−] HMEC-E6 cells grown in contract with growth factor–depleted rECM demonstrated evidence of apoptosis including the following: (1) nuclear condensation, (2) cell shrinkage (sh) and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements. In contrast, passage 11 p53[+] HMEC-LXSN controls (b) grown in contact with growth factor–depleted rECM did not demonstrate evidence of apoptosis at 14 d. Passage 11 p53[−] HMEC-E6 cells (c) and passage 11 p53[+] HMEC-LXSN controls (d) grown in collagen I/IV for 7 d did not demonstrate evidence of apoptosis by morphologic criteria. Light micrographs of passage 11 p53[−] HMEC-E6 cells grown in collagen I/IV supplemented with laminin (e) exhibited branching structures that terminated in spherical cell clusters. Electron micrographs of passage 11 p53[−] HMEC-E6 cells grown in collagen I/IV supplemented with laminin (g) demonstrated morphologic evidence of apoptosis including cell shrinkage (sh) and nuclear condensation (nu) at 7 d. Light micrographs of passage 11 p53[+] HMEC-LXSN vector controls grown in collagen supplemented with laminin demonstrated branching structures similar to those exhibited by early passage p53[−] HMEC-E6 cells (f) but did not demonstrate evidence of apoptosis by EM (h).

Figure 12. **Late passage p53⁻ HMEC-E6 cells exhibit decreased expression of integrin α3.** Early passage p53⁺ HMEC-LXSN controls (passage 10) (a and e), early passage p53⁻ HMEC-E6 cells (passage 10) (b and f), late passage p53⁺ HMEC-LXSN controls (passage 18) (c and g) and late passage p53⁻ HMEC-E6 cells (passage 21) (d and h) were grown on glass coverslips for 48 h and then stained by indirect immunofluorescence with mAb P1F2 against integrin α3 (a–d) or with P4C10 against β1-integrin (e–h).

10 μm   10 μm
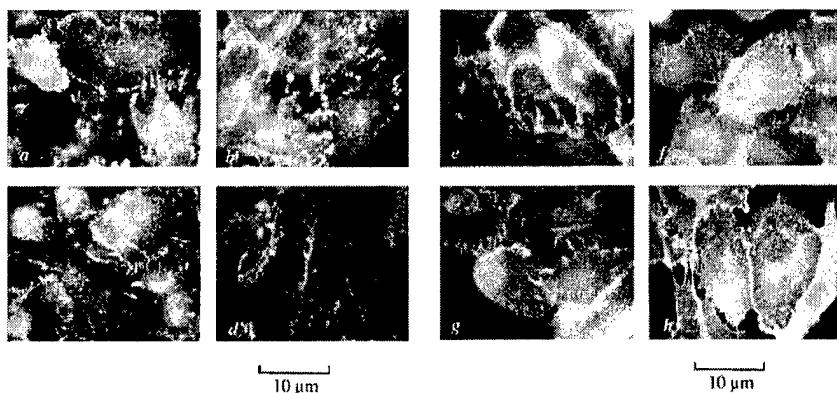
treated with α3- or β1-integrin blocking Abs demonstrated similar results (Fig. 14 b). These data show that interruption of α3/β1-integrin signaling by a blocking Ab inhibits rECM-mediated growth arrest in both p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6.

α3- and β1-integrin function-altering Abs inhibit rECM-mediated apoptosis of early passage p53⁻ HMEC-E6 cells

Early passage p53⁻ HMEC-E6 cells and early passage p53⁺ HMEC-LXSN controls were treated with either α3- or β1-integrin function-altering Abs or control mouse nonimmune IgG and grown in rECM. Electron micrographs of early passage p53⁻ HMEC-E6 cells and early passage p53⁺ HMEC-LXSN controls treated with either α3- or β1-integrin blocking Abs (clones P1B5 and JB1A, respectively) demonstrated disorganized cell aggregates without evidence of apoptosis at day 7 or 14 (Fig. 15; unpublished data). Early passage p53⁻ HMEC-E6 cells treated with β1-integrin blocking Abs also exhibited increased cell–cell separation (Fig. 15 b). Control p53⁻ HMEC-E6 cells treated with nonspecific mouse IgG or β1-integrin stimulatory Ab (clone B3B11) underwent apoptosis on day 7 (unpublished data). Control p53⁺ HMEC-LXSN controls treated with nonspecific mouse IgG did not exhibit apoptosis at day 7 (unpublished data). These results suggest that α3/β1-integrin-mediated

signal transduction may be critical for the induction of apoptosis in early passage p53⁻ HMEC-E6 cells grown in contact with rECM.

Discussion

p53 is a critical regulator of cell cycle control, and the high frequency with which p53 is functionally inactivated in human malignancy attests to its key role in preventing the clonal expansion of mutated cells (Hansen and Oren, 1997; Goetz et al., 2001). Although there is extensive experimental evidence demonstrating the importance of p53 as a tumor suppressor and in promoting differentiation, little is known about the fate of normal human cells that acutely lose p53 function in the context of rECM regulatory signals.

Abnormal p53 expression in benign breast tissue is associated with the subsequent development of breast cancer and may represent a very early event in breast carcinogenesis (Fabian et al., 1996; Levesque et al., 1998; Rohan et al., 1998). Interactions between mammary epithelial cells and ECM play a critical role in maintaining normal tissue homeostasis and are likely to be disrupted during breast carcinogenesis. In this report, we describe an in vitro system for investigating interactions between HMECs with suppressed p53 protein expression and rECM as a potential model of early mammary carcinogenesis.

Figure 13. **Localization of α3- and β1-integrin expression in p53⁺ HMEC-LXSN and p53⁻ HMEC-E6 cells using immunofluorescence microscopy.** Frozen section of early passage p53⁺ HMEC-LXSN controls (passage 11) (a and e), early passage p53⁻ HMEC-E6 cells (passage 11) (b and f), late passage p53⁺ HMEC-LXSN controls (passage 16) (c and g), and late passage p53⁻ HMEC-E6 cells (passage 21) (d and h) grown in rECM for 6 d, cryosectioned, and stained with monoclonal Abs to α3- (a–d) and β1- (e–h) integrins as described in Materials and methods. Integrins α3 and

30μm
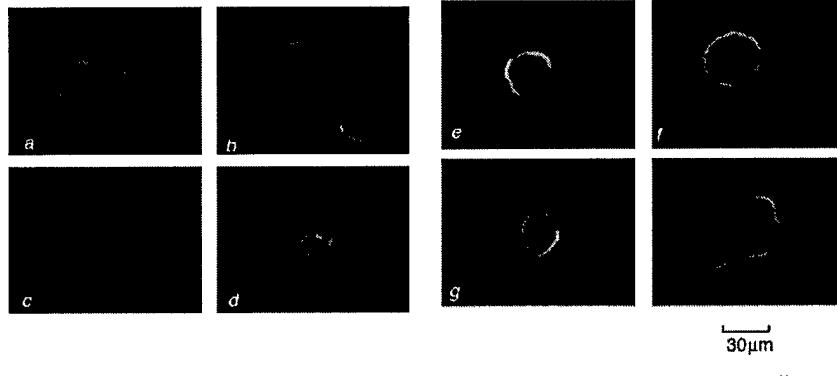
β1 were localized to the cell-rECM junction in early and late passage p53⁺ HMEC-LXSN cells and in early passage p53⁻ HMEC-E6 cells (a–c and e–g). In contrast, late passage p53⁻ HMEC-E6 cells demonstrated disorganized membrane and cytosolic staining (d).
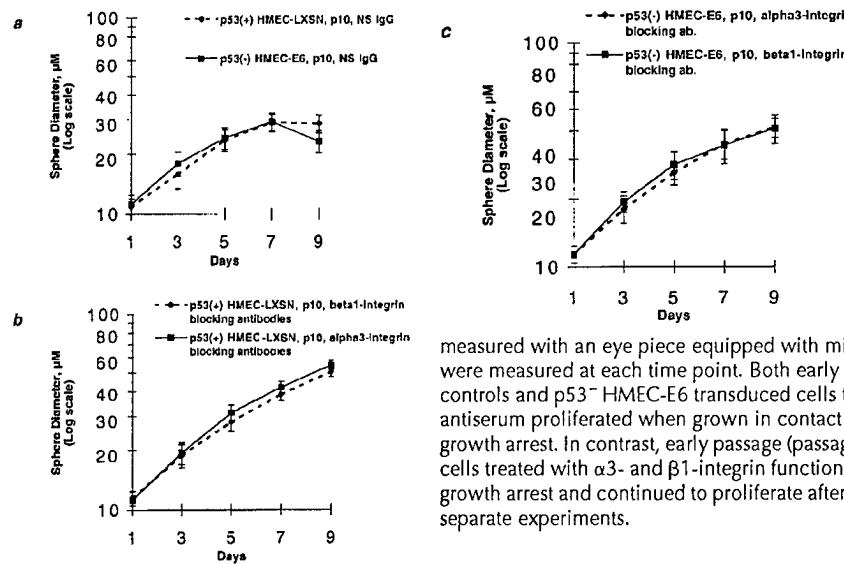
Figure 14. Treatment with α3- and β1-integrin function-altering Abs blocks rECM-mediated growth arrest in p53$^+$ and p53$^-$ HMECs. The mean diameter of spheres formed by p53$^+$ HMEC-LXSN controls, passage 10 (a and b) and p53$^-$ HMEC-E6 cells, passage 10 (a and c) treated with α3- or β1-integrin function-altering Abs (b and c) or nonimmune mouse IgG (a) is plotted as a function of days in culture. Cells were plated in rECM on day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with micrometer spindle. The 20 largest colonies were measured at each time point. Both early passage (passage 10) p53$^+$ HMEC-LXSN controls and p53$^-$ HMEC-E6 transduced cells treated with nonimmune mouse IgG control antiserum proliferated when grown in contact with rECM until day 7 and then underwent growth arrest. In contrast, early passage (passage 10) p53$^+$ HMEC-LXSN and p53$^-$ HMEC-E6 cells treated with α3- and β1-integrin function-altering Abs were resistant to rECM-induced growth arrest and continued to proliferate after day 7. These data are representative of three separate experiments.

Retrovirally mediated expression of HPV-16 E6 and AS ODNs were used to acutely inhibit p53 protein expression in HMECs (Figs. 1 and 5). The combination of these approaches allowed us to utilize both viral and nonviral methods to suppress p53. We observed that early passage p53$^+$ HMEC control cells underwent rECM-mediated growth arrest on day 6–7 and formed acinus-like structure (Figs. 3, 4, and 10). In contrast, early passage p53$^-$ HMEC-E6 cells and early passage p53$^-$ HMEC-AS cells proliferated until day 6 (Figs. 3, 4, and 6) and then underwent apoptosis on day 7 as evidenced by EM and by in situ TdT staining (Figs. 4 and 7; unpublished data). These observations suggest that the acute suppression of p53 function in HMECs by HPV-16 E6 and by AS ODNs may promote sensitivity to rECM-induced apoptosis.

ECM has been shown to provide essential signals for mammary epithelial cell survival and in their absence cells undergo apoptosis (Streuli et al., 1991; Strange et al., 1992; Pullan et al., 1996). The critical relationship between ECM signaling and p53 expression is highlighted by a recent report that ECM survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis (Ilic et al., 1998). However, there is significant evidence that growth arrest, differentiation, and survival signals may also promote apop-
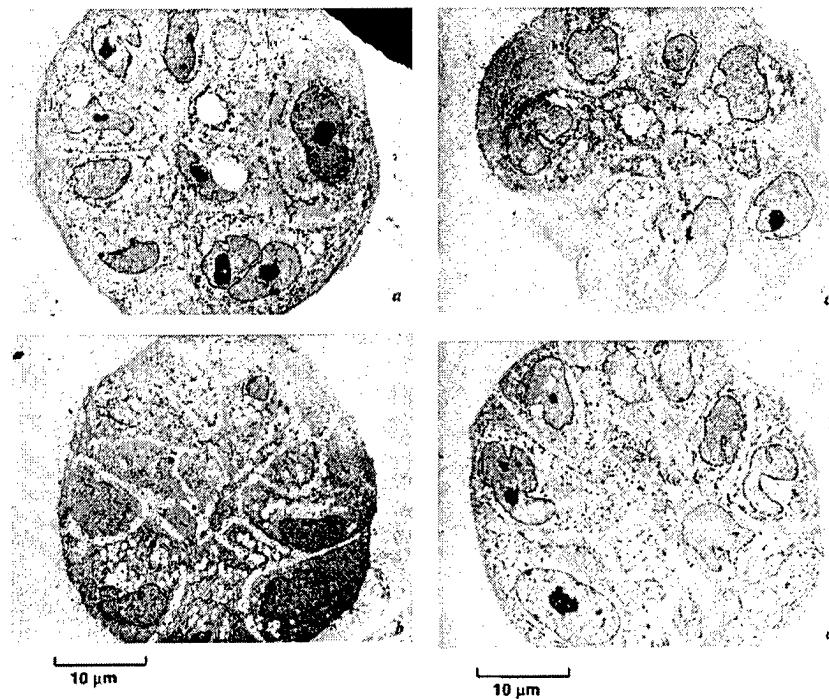
Figure 15. Inhibition of α3- and β1-integrin signaling blocks induction of rECM-mediated apoptosis. Electron micrographs of passage 10 p53$^-$ HMEC-E6 cells (a and b) and passage 10 p53$^+$ HMEC-LXSN controls (c and d) treated with 10 μg/ml α3- (a and c) or 20 μg/ml β1- (b and d) integrin blocking Abs and then grown in rECM for 7 d. Cells grew in multilayered colonies and did not growth arrest at day 6–7. There was no morphologic evidence of apoptosis. Evidence of lack of polarity included the following: (1) cells are not organized into spherical single layer structures, (2) a lumen was not present, and (3) vacuoles and nuclei were randomly distributed throughout the cytoplasm. Cells treated with β1-integrin blocking Abs exhibited cell–cell separation. There was no evidence of apoptosis.

10 μm

10 μm

tosis in genetically damaged cells (Seewaldt et al., 1995; Hong and Sporn, 1997; Mancini et al., 1997; Seewaldt et al., 1997b).

Our observation that the acute suppression of p53 in HMECs promotes sensitivity to apoptosis is consistent with several reports in human and mouse primary fibroblasts that the acute suppression of p53 results in apoptosis sensitivity (Hawkins et al., 1996; Wahl et al., 1996; Lanni et al., 1997). The mechanism by which the acute loss of p53 function might promote apoptosis is unknown. However, we observe that immediately after suppression of p53 by HPV-16 E6, HMECs exhibited a high percentage of tetraploid cells (14%) and an increased sensitivity to apoptosis. The tumor suppressor p53 is a critical component of the spindle checkpoint that ensures the maintenance of diploidy, and an increase in tetraploidy has been observed in cultured fibroblasts from p53-deficient mouse embryo fibroblasts (Cross et al., 1995). We speculate that it is possible that a loss in the spindle checkpoint might promote the induction of apoptosis when early passage p53$^-$ HMEC cells are exposed to either survival or growth arrest signals. Alternatively, p53 also plays an important role in differentiation, and therefore lack of p53 might result in cellular disregulation that promotes cell death. We observe that early passage p53$^-$ HMECs are sensitive to the induction of apoptosis by other agents that only promote growth arrest in p53$^+$ HMECs at equimolar concentrations. For example, we observed that although tamoxifen promotes G1/S-phase arrest in early passage p53$^+$ HMEC controls, tamoxifen promoted apoptosis in early passage p53$^-$ HMECs (Dietze et al., 2001; Seewaldt et al., 2001). Taken together, these observations provide evidence that the acute suppression of p53 might promote the induction of apoptosis when cells are exposed to growth arrest or survival signals.

Although early passage p53 HMEC-E6 cells underwent apoptosis when cultured in rECM, late passage cells were resistant to rECM-induced growth arrest, did not exhibit epithelial polarity, and failed to undergo apoptosis (Fig. 9). Loss of epithelial polarity is based on morphologic appearance by EM and lack of polarized expression of E-cadherin (Figs. 9 and 10). The development of apoptosis resistance correlated with the appearance of complex karyotypic abnormalities. Unlike early passage cells, late passage p53$^-$ HMEC-E6 cells continued to proliferate in rECM, formed multilayered aggregates of cells, and subsequently did not undergo apoptosis (Figs. 8 and 9). Based upon observations in our in vitro system, we propose that resistance to rECM-mediated growth arrest and polarity may promote resistance to apoptosis.

We investigated which component of rECM may be proapoptotic. Early passage p53$^-$ HMEC-E6 cells grown in collagen did not form acinar structures and did not undergo apoptosis (Fig. 11). Based on these observations, we speculate that the presence of collagen I/IV in a 1:1 ratio in our model system is not sufficient to induce apoptosis nor promote an acinar structure. When laminin was added to collagen I/IV gels, early passage p53$^-$ HMEC-E6 cells formed organized branched tubular structures that terminated in spherical cell clusters and underwent apoptosis (Fig. 11). Early passage p53$^-$ HMEC-E6 cells cultured in laminin sus-

pension, in the absence of a plastic substratum for adhesion, formed similar three dimensional structures and likewise underwent apoptosis. This suggests that contact with laminin is critical for the induction of apoptosis in early passage p53$^-$ HMEC-E6 cells.

Integrin α3β1 is a critical mediator of intracellular adhesion and an important receptor for laminin-5 (Xia et al., 1996; Kawano et al., 2001). Recently, α3/β1-integrin has been shown to play a potential role in the initiation apoptosis in T cells (Sato et al., 1999). Resistance to rECM-mediated apoptosis in late passage p53$^-$ HMEC-E6 cells was associated with altered expression of α3-integrin (Figs. 12 and 13). Redistribution of α3-integrin has been seen previously by other investigators in association with loss of mammary epithelial cell polarity in rECM culture and is consistent with our findings (Weaver et al., 1997). Treatment of early passage p53$^-$ HMEC-E6 cells with α3- and β1-integrin function-altering Abs blocked rECM-mediated growth arrest and inhibited the induction of apoptosis (Figs. 14 and 15). Taken together, these observations suggest an important role for α3/β1 signaling in rECM-mediated growth regulation and apoptosis.

Previous investigators have tested the ability of α3- and β1-integrin blocking Abs to mediate growth of breast cell lines in rECM and in collagen and fibrin gels (Howlett et al., 1995; Alford et al., 1998). HMT-3522, a nontumorigeneic breast cell line, demonstrated decreased proliferation in rECM culture when treated with either the inhibitory anti–α3-integrin Ab P1B5 or anti–β1-integrin Ab AIIB2 (Howlett et al., 1995). In contrast, we observed dysregulated proliferation when we treated HMECs with either the inhibitory anti–α3-integrin Ab P1B5 or anti–β1-integrin Ab JB1A. The induction of stimulatory or inhibitory functions by Abs directed to defined integrin subunits has been observed previously for both anti–β1- and anti–α3-integrin Abs (Lenter et al., 1993; Driessens et al., 1995; Lichtner et al., 1998) and is felt to be highly cell type specific. We hypothesize that utilization of nonimmortalized cells with low levels of α6β4-integrin expression may account for differences between our results and those obtained by Howlett et al. (1995).

In conclusion, we have shown that whereas p53$^+$ HMEC-LXSN cells undergo growth arrest and form polarized epithelium when grown in contact with rECM, p53$^-$ HMEC-E6 and p53$^-$ HMEC-AS cells that have acutely lost p53 function undergo apoptosis. Resistance to rECM-mediated growth arrest and polarity results in resistance to rECM-mediated apoptosis and is associated with altered expression of α3-integrin. Treatment of apoptosis-sensitive early passage p53$^-$ HMEC-E6 cells with either α3- or β1-integrin function-altering Abs results in loss of rECM-mediated growth arrest and resistance to rECM-mediated apoptosis. We suggest that sensitivity and resistance to rECM-mediated apoptosis in p53$^-$ HMECs is dependent on the ability to form a polarized epithelium and may require α3/β1-integrin signaling.

Materials and methods

Cell culture and media

stitute of Aging, Cell Culture Repository (Stampfer, 1985). HMEC strains AG11132 and AG11134 were established from normal tissue obtained at reduction mammoplasty, have a limited life span in culture, and fail to divide after ~20–25 passages. AG11132 and AG11134 cells exhibit a low level of estrogen receptor staining characteristic of normal mammary cells. AG11132 and AG11134 were at passage 8 at the time of receipt. HMECs were grown in standard medium: mammary epithelial cell basal medium (Clonetics) supplemented with 4 mg/ml bovine pituitary extract (CC4009; Clonetics), 5 mg/ml insulin (Upstate Biotechnology), 10 ng/ml EGF (Upstate Biotechnology), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 10^{-5} M isoproterenol (Sigma-Aldrich), and 10 mM Hepes buffer (Sigma-Aldrich). G418 (GIBCO BRL) containing standard medium was prepared by the addition of 300 mg/ml of G418 to standard medium. Cells were cultured at 37°C in a humidified incubator with 5% CO_2/95% air. Mycoplasma testing was performed as reported previously (Seewaldt et al., 1997a).

Retroviral transduction

The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence (provided by D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) has been described previously (Halbert et al., 1991; Demers et al., 1994). HMECs (passage 9) were plated in four T-75 tissue culture flasks (Corning) in standard medium and grown to 50% confluency. Transducing virions from either the PA317-LXSN16E6 or the control PA317-LXSN (without insert) retroviral producer line were added at a multiplicity of infection of 1:1 in the presence of 4 μg/ml polybrene (Sigma-Aldrich) to log-phase cells grown in T-75 flasks. The two remaining T-75 flasks were not infected with virus. After 48 h, the two flasks containing transduced cells and one flask with untransduced cells were passaged 1:3 (passage 10) and selected with standard medium containing 300 μg/ml G418. Cells were grown in G418 containing standard medium for 1 wk until 100% of control untransduced cells were dead. The transduction efficiency was high during selection, cells were passaged 1:3 at the completion of selection (passage 11), and cells were maintained in the absence of selection before immediately proceeding to apoptosis experiments. The fourth flask of unselected untransduced parental control cells was passaged in parallel with the selected transduced experimental and vector control cells. Parental AG11132 cells were designated HMEC-P, transduced cells expressing the HPV-16E6 construct were designated p53⁻ HMEC-E6, and vector control clones were designated p53⁺ HMEC-LXSN. All cells were maintained in standard medium after transfection in the absence of G418 selection to ensure that any chromosomal abnormalities or apoptosis resistance observed was not due to continued exposure to G418. All experiments were performed on mass cultures.

p53 oligonucleotides

The p53 AS oligonucleotide is an 18-mer targeting the region of the initiation codon (six base pairs immediately before the first and the first four coding codons): 5'-CGGCTCCTCCATGGCAGT-3'. This AS ODN has been used previously by several investigators to suppress p53 function (Bi et al., 1994; Capoulade et al., 2001). The p53 control ODN (5'-CGGCTC-CTCATGGCAGT-3') was chosen to be a scrambled sequence of the AS ODN to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content (Capoulade et al., 2001). In all ODNs, the first and the last three nucleotides were phosphorothiolate modified to increase stability in vitro.

Early passage p53⁺ HMEC-P parental cells were plated in T-75 plates in standard medium. After allowing 24 h for attachment, cell cultures were treated for 72 h with either 0.1 μM active or scrAS p53 ODNs. The culture medium was replaced by new standard medium containing fresh ODNs every 24 h. Western blot analysis was performed to confirm suppression of p53 expression as described below. The resulting film images were digitized and quantitated using Eastman Kodak, Co. 1D image analysis software.

rECM culture was as follows: cells were trypsinized, and ~10⁴ cells were resuspended in 100 μl rECM containing 0.1 μM of either active or p53 scrAS ODN on ice. rECM cultures were prepared as described below. rECM cultures were overlayed with standard medium containing 0.1 μM of either active or scrambled p53 ODNs. Overlay medium was changed every 24 h to ensure a fresh supply of ODNs. The diameter of the growing colonies was determined, and cell colonies were prepared for EM as described below. To measure p53 protein expression in cells grown in rECM culture, colonies were released from the matrix by 60-min incubation at 37°C with dispase (5,000 U/ml caseinolytic activity; Collaborative Research). Released cells were washed once using ice-cold PBS with 5 mM EDTA and twice with PBS alone. The resulting pellet was tested for p53 protein expression by Western blot analysis as described below.

Western blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt et al., 1995, 1997b). p53 expression was detected using a 1:100 dilution of mouse anti-human p53 Ab-2 (Oncogene Research Products) and detected by ECL Western blotting detection reagents (Amersham Pharmacia Biotech) as described by the manufacturer.

Northern blot analysis

RNA was extracted with guanidium isothiocyanate and subjected to Northern blotting in formaldehyde denaturing gels as described previously (Seewaldt et al., 1995). 10 mg of RNA were loaded per lane. Molecular probes used in the Northern blot analysis were as follows: the human p53 probe was a 1.9-kb BamH1 fragment (Seewaldt et al., 1997b), and the 36B4 probe was a 700-bp PstI fragment that was used as a loading and transfer control probe (Seewaldt et al., 1995).

HMEC culture in reconstituted ECM

Mammary epithelial cells were grown in rECM by methods developed by Bissell and others (Folkman and Moscona, 1978; Howlett et al., 1994; Roskelley et al., 1994; Seewaldt et al., 1997b). 100 μl of rECM (Matrigel™; Collaborative Research) or growth factor–depleted rECM (growth factor–reduced Matrigel™; Collaborative Research) were added per well to a 48-well plate and allowed to gel at 37°C for 20 min. p53⁻ HMEC-E6–transduced cells and p53⁺ HMEC-LXSN vector controls were trypsinized, counted, and pelleted in a sterile microcentrifuge tube. Approximately 10⁴ cells were resuspended in 100 μl rECM on ice, gently overlaid on the initial undercoating of ECM, and allowed to gel at 37°C for 20 min. Standard medium was then added, and wells were inspected to ensure an equal distribution of cells in each well. Cells were grown for 5–14 d in culture. Medium was changed daily.

For integrin-blocking experiments, ~10⁴ p53⁺ HMEC-LXSN vector control cells (passage 11) or p53⁻ HMEC-E6 cells (passage 10) were pelleted and resuspended in 100 μl standard medium containing either Abs to α3- and β1-integrins (Chemicon International) or control nonimmune mouse IgG for 15 min at room temperature (RT). Final concentration of α3-integrin blocking Ab (CDW496, clone P1B5) was 10 μg/ml, β1-integrin blocking Ab (CD29, clone JB1A) was 20 μg/ml, and β1-integrin stimulatory Ab (CD29, clone B3B11) was 10 μg/ml. 100 μl rECM was added to the cell suspension, gently mixed, and overlaid as described above. 1 ml standard medium containing the above respective concentration of blocking Ab was added to each well and changed every other day. Cells were grown for 5–9 d in rECM culture.

Collagen/laminin morphogenesis assays

Collagen and collagen/laminin gels were prepared by a modification of methods developed by Alford et al. (1998). Collagen type I (Sigma-Aldrich) and human placental collagen type IV (Sigma-Aldrich) were solubilized in 0.018 N acetic acid for a final concentration of 3 mg/ml each. Three parts collagen type I were mixed with one part collagen type IV. The collagen I/IV solution was neutralized by mixing 8 vol of collagen solution with 1 vol of sterile PBS and 1 vol of sterile 0.1 M NaOH for a final pH of 7.4. 100 μl of neutralized collagen I/IV solution were added per well to a 48-well plate and allowed to gel at 37°C for 20 min. Approximately 10⁴ early passage p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN controls were resuspended in 100 μl neutralized collagen I/IV solution on ice, gently overlaid on the initial undercoating of collagen, and allowed to gel at 37°C for 20 min. Standard medium was then added, and wells were inspected to ensure an equal distribution of single cells suspended in each well. Cells were grown for 5–9 d in culture and then prepared for EM as described previously (Seewaldt et al., 1997b). For collagen/laminin gels, nine parts collagen I/IV were added to one part human placental laminin (Sigma-Aldrich), and gels were prepared as above. Laminin cultures were prepared as follows: 48-well plates were coated with 100 μl neutralized collagen I/IV solution and baked at 65°C for 24 h. This was repeated three times. 100 μl of human placental laminin were added per well and baked at 50°C until the laminin solution hardened. This was repeated three times. Approximately 10⁴ early passage p53⁻ HMEC-E6 cells were suspended in a 1:1 mixture of standard medium and human placental laminin. Cells were grown for 7 d and prepared for EM as previously described (Seewaldt et al., 1997b).

Transmission EM

p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN vector control cells were grown in contact with rECM as described above, and EM was preformed as described previously (Seewaldt et al., 1997b).

Cell growth determination in rECM culture

Cell growth was determined by the following criteria: the size of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. For both p53+ HMEC-LXSN vector controls and p53⁻ HMEC-E6–transduced cells, the 20 largest colonies were measured.

Detection of apoptosis by in situ TUNEL

p53⁻ HMEC-E6 cells and p53+ HMEC-LXSN vector control cells were grown in contact with rECM as described above for 5–9 d. Cells were then fixed in PBS with 10% formalin and embedded in paraffin. Sections were deparaffinized and quenched in methanol containing 2.1% hydrogen peroxide. Antigen retrieval was achieved by placing slides in 10 mM citric acid at 95°C for 10 min. Nuclear proteins were stripped with 20 μg/ml proteinase K, and slides were washed in deionized water. Positive controls were immersed in DN buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 4 mM magnesium chloride, 0.1 mM DTT) for 5 min at RT and then incubated with DNAseI (Roche) in DN buffer for 10 min at RT. Negative controls were treated with 5% FBS. All samples were immersed in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1.0 mM cobalt chloride) for 5 min at RT. Sections were covered with TdT/Bio-14-dATP solution (800 μl TdT buffer containing 120 U terminal transferase and 50 nM Bio-14-dATP [GIBCO BRL]), incubated for 1 h at RT, and then the reaction was terminated with PBS. Sections were blocked with 2% BSA for 10 min at RT and treated with ABC solution (Elite). Sections were allowed to complex on ice for 30 min, incubated for 30 min at 37°C, and were washed in PBS. Sections were stained with DAB (2% nickel chloride, 0.1% hydrogen peroxide) for 3 min at RT and counterstained.

E-cadherin immunostaining

Early and late passage p53⁻ HMEC-E6 cells and p53+ HMEC-LXSN vector control cells were grown in rECM as described for 6 d and embedded in OCT (Miles). Cells were snap frozen, and 5-μm sections were obtained. Sections were fixed in for 30 min at RT with 3.7% formaldehyde in PBS and were blocked with 0.5% heat-denatured BSA (HD-BSA) in PBS for 1 h at RT. Cells were then incubated for 30 min with mouse anti–human E-cadherin Ab (BD Signal Transduction Laboratories), diluted in PBS with 0.5% HD-BSA for 30 min at RT, and then washed six times with PBS at RT. For Immunofluorescence, cells were incubated with FITC-conjugated goat anti–mouse Ab at a 1:200 Ab dilution (Santa Cruz Biotechnology, Inc.) in PBS with 0.5% HD-BSA for 30 min at RT and washed. Sections were mounted in 30% glycerol in PBS and visualized for immunofluorescence using a ZEISS LSM 410 fluorescence microscope.

Immunodetection of integrin expression

Early and late passage p53⁻ HMEC-E6 cells and p53+ HMEC-LXSN vector control cells were grown on glass coverslips for 48 h in standard medium. Cells were fixed in for 20 min at RT with 2% formaldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose at pH 7.2, permeabilized with 0.1% Triton X-100 for 10 min at RT, and blocked with 0.5% HD-BSA in PBS for 1 h at RT. Cells were then incubated with a primary Ab diluted in PBS with 0.5% HD-BSA for 1 h at RT and washed six times with PBS at RT. Abs against integrin subunits α2 (P1H5), α3 (P1F2, P1B5), and β1 (P4C10) were a gift from William Carter (Fred Hutchinson Cancer Research Center) and have been described previously (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990a,b). Abs against integrin α6 (GoH3) and β4 (3E1) were obtained from Chemicon International. For Immunofluorescence, cells were incubated with either FITC-conjugated goat anti–mouse Ab at a 1:1,500 dilution or goat anti–rat Ab at a 1:4,000 dilution (Dako) in PBS with 0.5% HD-BSA for 1 h at RT and washed. Sections were mounted in a solution containing 25 mg/ml of 1,4-diazobicyclo-(2,2,2)octane in glycerol and visualized for immunofluorescence using a ZEISS LSM 410 fluorescence microscope.

For rECM culture, early and late passage p53⁻ HMEC-E6 cells and p53+ HMEC-LXSN vector control cells were grown in contact with rECM for 6 d in standard medium. Cells were embedded in OCT, snap frozen, and sectioned as described above. Sections were fixed in for 20 min at RT with 2% formaldehyde in 0.1 M sodium cacodylate, 0.1 M sucrose, pH 7.2, and blocked with 0.5% HD-BSA in PBS for 1 h at RT. Sections were stained with Abs against integrin subunits α3 (P1F2) and β1 (P4C10) and visualized as described above.

Cytogenetic analysis of early passage transduced and parental HMECs

Cultures of p53+ HMEC-P parental cells (passage 10), p53+ HMEC-LXSN vector controls (passage 10), p53⁻ HMEC-E6 cells (passage 10), and p53⁻

HMEC-E6 cells (passage 18) were checked for sufficient numbers of dividing cells and exposed to colcemid (GIBCO BRL) at a final concentration of 0.01–0.02 μg/ml for 2–3 h. Subsequently, the cells were released from flasks by trypsinization, exposed to hypotonic solution, and fixed as described previously (Mrózek et al., 1993). Chromosome preparations were made, and after appropriate aging slides were subjected to SKY, a method that enables simultaneous display of all human chromosomes in different colors (Schröck et al., 1996). Additional slides were also stained with DAPI (Vector Laboratories) alone. For SKY, the slides were hybridized with the SKY probe mixture containing combinatorially labeled painting probes for each of the autosomes and sex chromosomes (Applied Spectral Imaging) for 42–45 h at 37°C. The hybridization and detection procedures were performed according to the manufacturer's protocol (Applied Spectral Imaging), and chromosomes were counterstained with DAPI in antifade solution. The multicolor hybridizations were visualized with the SpectraCube SD 200 system (Applied Spectral Imaging) mounted on the ZEISS Axioplan 2 epifluorescence microscope using a custom-designed optical filter (SKY-1; Chroma Technology). The DAPI images of all metaphase cells were acquired using a DAPI-specific optical filter. Spectral analysis and classification were performed using SkyView 1.2r visualization and analysis software (Applied Spectral Imaging). The assignment of breakpoints in structural abnormalities was made on comparison of images of SKY classified chromosomes with the images of the same chromosomes stained with DAPI that were inverted electronically and contrast enhanced by SkyView 1.2r software. Karyotypic abnormalities were classified according to the recommendations of the International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

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CBP/p300 induction is required for retinoic acid sensitivity in human mammary cells

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Abstract

The coactivators CBP and p300 are recruited by retinoic acid receptors (RARs) during retinoid mediated transcriptional regulation. To assess the role of CBP/p300 in all-*trans*-retinoic acid (ATRA)-mediated growth arrest in mammary epithelial cells, two systems were tested: (1) ATRA resistant MCF-7 cells were transduced with a functional RAR-β2; (2) normal human mammary epithelial cells (HMECs) were transduced with a pan-RAR dominant negative, RAR-α403. Expression of RAR-β2 in MCF-7 cells resulted in increased sensitivity to ATRA-induced growth arrest and correlated with induction of CBP/p300 mRNA and protein. Inhibition of RAR function in HMECs resulted in resistance to ATRA-induced growth arrest and loss of CBP/p300 induction. Antisense suppression of CBP/p300 in HMECs resulted in decreased retinoic acid response element reporter *trans*-activation and decreased ATRA-mediated growth arrest. Thus, in human mammary epithelial cells, CBP/p300 were both modulated by an ATRA signaling pathway and were required for a normal response to ATRA.
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CBP and p300 are important regulators of transcriptional activation and are integrators of many signaling pathways that control transcription [1,2]. Retinoic acid receptors (RARs), and other nuclear hormone receptors, are among the many transcriptional regulators that interact with CBP and p300. All-*trans*-retinoic acid (ATRA) dependent growth control, differentiation, and apoptosis depend on the presence of CBP and p300 in F9 cells [3]. Fibroblasts derived from p300 null mice have defective retinoid signaling [4]. CBP and p300 are thought to be present in limiting amounts in the cell. The ability of various signaling pathways to regulate transcription depends on the particular pathway's ability to compete for CBP and p300 [1,2,5].

The activity of CBP and p300 can be altered by phosphorylation and binding of proteins such as human papillomavirus-16 E6 protein, SV40 large T antigen, and adenovirus E1A protein [1,2,6]. CBP levels have been

shown to be altered in Rubinstein–Taybi syndrome (RTS) in which a loss of activity in one CBP allele is observed [7,8]. RTS is characterized by abnormal pattern formation, mental retardation, and an increased risk of cancer. RTS is also associated with premature breast development [9]. Mice with either one or both alleles of CBP and p300 inactivated have been constructed [4,7,8]. Homozygous loss of either CBP or p300 function is lethal, as is loss of one allele of both CBP and p300. This indicates that both CBP and p300 are essential and that, to some extent, they have overlapping functions. Heterozygous loss of CBP in mice results in pathology similar to RTS [11]. Similarly, the loss of one allele of p300 also results in developmental defects [4]. Recently, p300, but not CBP, was shown to be transcriptionally regulated by BRCA1 in breast cancer cell lines [10]. In these cell lines, BRCA1 downregulated p300 mRNA and protein. The decrease in p300 resulted in inhibition of estrogen receptor-α (ERα) activity. ERα expression was rescued by overexpression of either p300 or CBP in these cells.

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Progressive loss of RAR-β2 expression has been observed during epithelial carcinogenesis [11–16]. Loss of RAR-β2 expression has been observed in breast cancer as well as in lung, prostate, cervical, esophageal, gastric, and oral carcinomas [17–21]. This loss is hypothesized to be the result of both genetic and epigenetic events. Two mechanisms have been proposed: (1) loss of heterozygosity; (2) promoter methylation [17,22,23]. Loss of RAR-β2 expression is hypothesized to be important for breast carcinogenesis, since RAR-β2 acts to regulate mammary tumor growth in vivo and in vitro and acts as a tumor suppressor in breast cancer [13–16]. Furthermore, inhibition of retinoid signaling by the dominant negative RAR, RAR-α403 (α403), in normal human mammary epithelial cells (HMECs) results in loss of extracellular matrix-regulated growth control and polarity [15].

CBP and p300 are also hypothesized to be tumor suppressors [1,2,24]. Since CBP and p300 are required for transcriptional regulation by retinoids in F9 cells [3], this raises the possibility that the regulation of gene expression by retinoids could itself be regulated by modulation of CBP and/or p300 activity. It has been shown recently that 9-cis-retinoic acid treated HSC-3 cells expressed in increased levels of CBP and p300 protein [25] and that ATRA modulates CBP and p300 protein levels in human mammary epithelial cells [16]. Thus, CBP/p300 and RAR-β2 could act in a concerted manner to suppress tumor growth. Using human mammary epithelial cells, we have investigated the relationship between CBP and p300 expression and sensitivity to ATRA mediated: (1) trans-activation of a retinoic acid response element (RARE)-driven CAT reporter; (2) growth regulation.

Materials and methods

Materials. All chemicals were obtained from Sigma, cell culture reagents and DNA primers were from Gibco-BRL, and cell culture plasticware was from Corning unless otherwise noted. A 1.0 mM ATRA stock solution was prepared in ethanol and stored in opaque tubes at −70 °C. ATRA stocks were only used under reduced yellow lighting. Control cultures received equivalent volumes of ethanol.

Cell culture conditions and retroviral transduction. Construction of the LXRARβ2SN and LXRARα403SN retroviral vectors has been previously described [14,15]. The MCF-7 variant used is retinoid-resistant and does not express RAR-β2 [14]. Expression of a functional RAR-β2 in these retinoid-resistant MCF-7 cells (MCF7-β2) resulted in increased sensitivity to ATRA-mediated: (1) growth inhibition; (2) trans-activation of a RARE as previously described [14]. MCF-7 cells transduced with the empty LXSN retroviral vector were used as controls (MCF7-SN) [14]. MCF-7 cells were grown in supplemented α-MEM as previously described [14]. All transduced MCF-7 cells were routinely maintained in this medium plus 1.0 mg/ml G418 except for the last two passages before experimental use. Growth media were not processed to remove endogenous retinoids.

HMECs were a gift of Martha Stampfer and were derived from reduction mammoplasty [15]. RAR-α403 is a truncated RAR-α with dominant-negative activity against all RAR isoforms (-α, -β, and -γ) [15]. As previously described, expression of the RAR-α403 in HMECs (HMEC-α403) decreased sensitivity to ATRA-mediated growth inhibition and trans-activation of a RARE [15]. HMECs transduced with the empty LXSN retrovirus were used as controls (HMEC-SN) [15]. Culture conditions and selection were as previously described [15]. Growth media were not processed to remove endogenous retinoids.

Northern blotting. Northern blotting was carried out as previously described [14,15].

SDS–PAGE and Western blotting. Three T-75 flasks of each cell type were grown to 50% confluency, treated for 24 h with 1.0 μM ATRA, harvested as previously described [16], and stored in 35 μl aliquots at −70 °C until use. The lysate was separated by 6% SDS–PAGE and transferred to a PVDF membrane which was blocked overnight with 10% BSA (w/v) dissolved in Tris buffered saline with 0.1% Tween 20 (TTBS). The blocked membrane was incubated with either 1/200 CBP C1 or 1/300 p300 N15 antibody (Santa Cruz) in TTBS, washed in TTBS, incubated with 1/6000 donkey-HRP conjugated secondary antibody (Santa Cruz), washed, and developed with SuperSignal West Pico reagent (Pierce). The film image was digitized and quantitated using Kodak1D software (Kodak).

Transient expression assay. CAT transient expression assays were carried out as previously described [20] with the pRRE4-tk CAT reporter plasmid. This plasmid contains four copies of the RARE from the promoter region of the hRARβ gene.

Cell growth curves and FACS. Growth curves and FACS analysis were performed as previously described [14,15,26].

Semiquantitative RT-PCR. First-strand cDNA was obtained from either 3 μg (MCF-7) or 5 μg (HMEC) of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) in a final volume of 20 μl. Amplification of the 272 bp CBP fragment was performed with previously reported primers, forward 5′-TCAGTC AACATCTCCTTCGC-3′ and reverse 5′-TGTTGAACATGAGCCA GACG-3′ [27]. Primers used to amplify the 2.7 kb p300 fragment were also previously reported [28]. The forward and reverse sequences, respectively, were 5′-AATGATGAGTGAAAATGCCAGTGTGCC C-3′ and 5′-GCAGTCGCTTGGGCTTGGGTAT-3′. Preliminary PCR was carried out to determine a cycle number that gave linear amplification.

The conditions used to amplify CBP from MCF-7 cDNA were as follows: (1) Each PCR contained 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 1 mM dNTPs, 200 nM primers, 1.25 U recombinant Taq polymerase (Roche), and 2.0 μl cDNA in a final volume of 50 μl. (2) Following an initial denaturation at 94 °C for 4 min, 28 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s were carried out with a final extension at 72 °C for 5 min.

Amplification of p300 from MCF-7 cDNA was performed using the following conditions: (1) Each PCR contained 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 1.0 mM dNTPs, 100 nM primers, and 2.0 μl cDNA in a final volume of 50 μl. (2) After an initial denaturation at 94 °C for 4 min, 2.0 U recombinant Taq polymerase (Roche) was added to each reaction. (3) Amplification was continued for 26 cycles at 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 3 min with final extension at 72 °C for 5 min.

CBP amplification with HMEC cDNA used the CBP MCF-7 conditions with the following changes: (1) Each reaction contained 20 mM Tris (pH 8.4), 1 mM MgCl₂, 50 mM KCl, 1 mM dNTPs, 100 nM primers, 1.25 U recombinant Taq polymerase (Roche), and 4.0 μl cDNA. (2) The cDNA was amplified using 29 cycles and the 58 °C incubation was for 30 s.

The p300 HMEC cDNA amplification used the conditions for p300 MCF-7 cDNA with the following changes: (1) Each reaction contained 75 nM primers. (2) Amplification was continued for 29 cycles. β-Actin was amplified as a positive control using forward (5′-GCTCGT CGTCGACAACGGCTC-3′) and reverse (5′-CAAACATGATCTGG GTCATCTTCTC-3′) primer sequences.

Amplification of β-actin from MCF-7 cDNA was carried out for 20 cycles and done according to the above-mentioned reaction and thermal cycling conditions used for MCF-7 CBP cDNA. The conditions for HMEC β-actin cDNA amplification were exactly the same as those for HMEC CBP cDNA amplification, except that 19 amplification cycles were used. The CBP- and p300-PCR products were visualized on 1.2% and 1% agarose/ethidium bromide gels, respectively. Gel images were obtained using an Image Station 440 (Kodak). The products were quantified using Kodak 1D software (Kodak). All PCRs were done in triplicate.

Antisense suppression of CBP and p300. CBP was suppressed in HMECs by the addition of the antisense (AS)-sequence 5′-CAC TTCAGGTTTCTTTTCATCC-3′. An inactive, scrambled (Scr) sequence 5′-ATTCTCATCATCGTCTTCGTTC-3′ was used as the
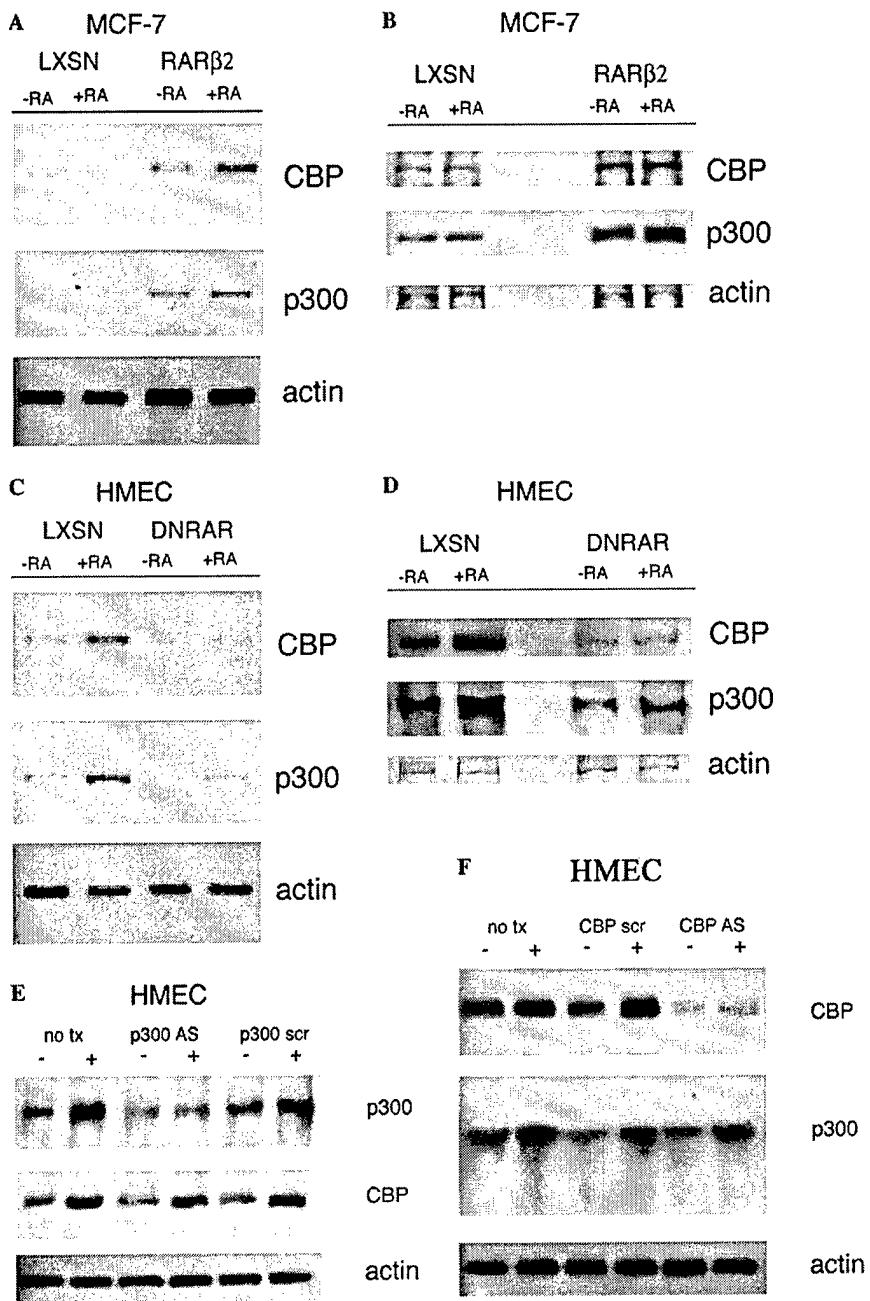
Fig. 1. Modulation of CBP/p300 expression in MCF-7 and HMEC cells by retinoid signaling and AS-ODNs. (A) Expression of CBP/p300 mRNA in MCF7-β2s (RAR-β2) and -SN controls (LXSN). (B) Expression of CBP/p300 protein in MCF7-β2s (RAR-β2) and -SN controls (LXSN). (C) Expression of CBP/p300 mRNA in HMEC-α403s (DNRAR) and in HMEC-SN controls (LXSN). (D) Expression of CBP/p300 protein in HMEC-α403s (DNRAR) and in HMEC-SN controls (LXSN). (E) Expression of CBP/p300 protein in AS-p300 treated HMEC-SNs. (F) Expression of CBP/ p300 protein in AS-CBP treated HMEC-SNs. Cells were treated with or without 1.0 μM ATRA for 48 h. HMECs were treated for 4 days with either AS-ODNs or Scr-ODNs.

negative control. Similarly, p300 was suppressed by the addition of the AS-sequence 5′-TTGTTGGTGGTGTAGGTGTC-3′ and the inactive, Scr-sequence 5′-TTTAGTGCGTTGGTGGTGTG-3′ was used as the negative control. Oligodeoxynucleotides (ODNs) were added to the growth media at a final concentration of 1 μM. Fresh ODNs were added every day. All nucleotides were phosphorothioate modified on the second, third, and last three bases. Cells were tested after 4 days of treatment.

Results

ATRA and RARs regulate CBP and p300 mRNA and protein expression

Expression of RAR-β2 in MCF-7 cells resulted in ATRA mediated induction of both CBP and p300 mRNA levels after treatment with 1 μM ATRA for 24 h (Fig. 1A, Table 1A). There was a 3.2-fold increase in the levels of CBP mRNA and a 2-fold increase in p300 mRNA levels. In MCF7-SNs treated with 1.0 μM ATRA, a decrease in both CBP and p300 mRNA was observed. However, the decrease was not significant

($p > 0.01$). Introduction of RAR-β2 into MCF-7 cells was not associated with a significant change in CBP mRNA levels ($p > 0.01$) but was associated with a 2.6-fold increase in p300 mRNA.

Similarly, expression of RAR-β2 in MCF-7 cells also resulted in ATRA mediated induction of both CBP and p300 protein levels (Fig. 1B, Table 1A). There was a 2.3-fold increase in CBP and a 2.6-fold increase in p300 protein levels in MCF7-β2s treated with 1.0 μM ATRA. ATRA treated MCF7-SNs exhibited a small increase in CBP that was not significant ($p > 0.01$) and no increase in p300 protein levels. As it was observed for mRNA, introduction of RAR-β2 had no effect on CBP protein levels, but p300 protein showed a 1.6-fold increase.

CBP and p300 mRNA levels were both decreased in HMEC-α403s as compared to HMEC-SNs (Fig. 1C, Table 1B). There was a 2.0-fold decrease in the level of CBP mRNA while there was a 2.3-fold decrease in the level of p300 mRNA. However, these changes were not significant ($p > 0.01$). When treated with 1.0 μM ATRA, HMEC-SNs showed a 12- and 28-fold increase in the amount of CBP and p300 mRNAs, respectively.

Table 1
Relative CBP and p300 expression in ATRA-treated transduced MCF-7 cells (A) and HMECs (B) or ATRA- and AS-p300- (C) and AS-CBP-treated HMECs (D)

(A)	MCF7-SN		MCF7-β2	
1 μM ATRA	(−)	(+)	(−)	(+)
CBP				
Protein	0.45 ± 0.06	0.55 ± 0.09	0.44 ± 0.08	$1.0 \pm 0.0^{*\wedge}$
mRNA	0.36 ± 0.2	0.20 ± 0.05	0.31 ± 0.1	$1.0 \pm 0.0^{*\wedge}$
p300				
Protein	0.24 ± 0.04	0.24 ± 0.03	$0.38 \pm 0.04^{*}$	$1.0 \pm 0.0^{*\wedge}$
mRNA	0.20 ± 0.05	0.072 ± 0.03	$0.51 \pm 0.04^{*}$	$1.0 \pm 0.0^{*\wedge}$

(B)	HMEC-SN		HMEC-α403	
1 μM ATRA	(−)	(+)	(−)	(+)
CBP				
Protein	0.63 ± 0.10	$1.0 \pm 0.0^{*\wedge}$	$0.31 \pm 0.07^{*}$	$0.28 \pm 0.07^{*}$
mRNA	0.082 ± 0.07	$1.0 \pm 0.0^{*\wedge}$	0.041 ± 0.06	0.082 ± 0.03
p300				
Protein	0.66 ± 0.03	$1.0 \pm 0.0^{*\wedge}$	$0.064 \pm 0.06^{*}$	$0.37 \pm 0.1^{*\wedge}$
mRNA	0.036 ± 0.05	$1.0 \pm 0.0^{*\wedge}$	0.016 ± 0.03	0.053 ± 0.02

(C) p300 ODN	HMEC-SN None		AS		Scr-AS	
1 μM ATRA	(−)	(+)	(−)	(+)	(−)	(+)
CBP protein	0.25 ± 0.06	$0.79 \pm 0.03^{*+}$	0.13 ± 0.02	$0.34 \pm 0.05^{\wedge+}$	0.21 ± 0.05	$1.0 \pm 0.0^{*\wedge+}$
p300 protein	0.26 ± 0.04	$0.68 \pm 0.06^{*+}$	$0.063 \pm 0.02^{*+}$	$0.061 \pm 0.02^{*+}$	$0.33 \pm 0.04^{\wedge}$	$1.0 \pm 0.0^{*\wedge+}$

(D) CBP ODN	HMEC-SN None		AS		Scr-ODN	
1 μM ATRA	(−)	(+)	(−)	(+)	(−)	(+)
CBP protein	0.79 ± 0.07	$0.98 \pm 0.04^{*+}$	$0.072 \pm 0.06^{*}$	$0.13 \pm 0.05^{*}$	0.58 ± 0.1	$0.97 \pm 0.04^{*+}$
p300 protein	0.40 ± 0.1	$1.0 \pm 0.0^{*+}$	$0.20 \pm 0.08^{*}$	$0.56 \pm 0.03^{\wedge+}$	$0.17 \pm 0.08^{*}$	$0.57 \pm 0.03^{\wedge+}$

HMEC-SN controls, HMEC-α403s, MCF7-SN controls, and MCF7-β2s were treated for 4 days with the indicated AS-ODN and then with the addition of 1.0 μM ATRA for the last 24 h. The relative levels of CBP/p300 protein levels were determined by Western blot analysis and quantitated using Kodak 1D Image Analysis Software. These data are means of three separate experiments ($^{*}/^{\wedge}$ = protein/mRNA level of CBP/p300 significantly different for $p < 0.01$ when (1) compared to untreated controls (*), (2) compared to ATRA treated controls ($^{\wedge}$), or (3) ATRA untreated and treated, ODN treated cells were compared ($^{+}$)).

HMEC-α403s treated for 24 h with 1.0 μM ATRA showed an induction in the level of CBP, 2.0-fold, and p300, 3.3-fold, mRNA that was not significant ($p > 0.01$).

Both CBP and p300 protein levels were also decreased in HMEC-α403s as compared to HMEC-SNs (Fig. 1D, Table 1B). There was a 2.2-fold decrease in the level of CBP while there was a 10-fold decrease in the level of p300. When treated with 1.0 μM ATRA, HMEC-SNs showed a 1.6- and 1.5-fold increase in the amount of CBP and p300 protein, respectively. Consistent with the lack of mRNA induction, ATRA treated HMEC-α403s showed no significant change ($p > 0.01$) in the amount of CBP protein (Fig. 1D, Table 1B). However, there was a 5.8-fold induction of p300 protein.

Antisense suppression of p300 and CBP in HMECs

Treatment of HMEC-SNs with AS- or Scr-p300 resulted in a 4.1-fold decrease and no significant change ($p > 0.01$) in p300 protein expression, respectively (Fig. 1E, Table 1C). Similar to p300, when HMEC-SNs were treated with AS-CBP or Scr-CBP, the CBP protein content showed a 4.8-fold decrease and no significant change ($p > 0.01$), respectively (Fig. 1F, Table 1D). CBP protein expression was not significantly ($p > 0.01$) altered by AS-p300 or by Scr-p300 (Fig. 1E, Table 1C). p300 protein levels were also not significantly ($p > 0.01$) altered by AS-CBP or Scr-CBP (Fig. 1E, Table 1D).

Scr-p300 had no significant effect on CBP protein levels with or without ATRA treatment (Fig. 1E, Table 1C). Unexpectedly, Scr-CBP treatment had the same effect on p300 protein levels as seen with AS-CBP treatment (Fig. 1F, Table 1D).

AS-p300 and AS-CBP completely blocked the ATRA mediated induction of themselves and had no effect on the ATRA mediated induction of each other (Tables 1C and D). As a result of decreased baseline levels of CBP from AS-p300 treatment and of p300 from AS-CBP treatment, ATRA treatment of HMEC-SNs in the presence of AS-p300 or -CBP gave comparable CBP/decreased p300 or decreased CBP/comparable p300 levels, respectively, when compared to HMEC-SNs that had not been treated with either ATRA or AS-ODN.

Antisense suppression of CBP and p300 modulates ATRA sensitivity in HMECs

Antisense suppression of CBP or p300 decreased ATRA induced expression of a CAT reporter driven by the RAR-β2 P2 promoter. CAT reporter activity was decreased by about 6.0- and 4.5-fold, respectively, in HMEC-SNs treated with AS-CBP and 0.1 or 1.0 μM ATRA (Fig. 2A). AS-p300 had a nearly identical effect
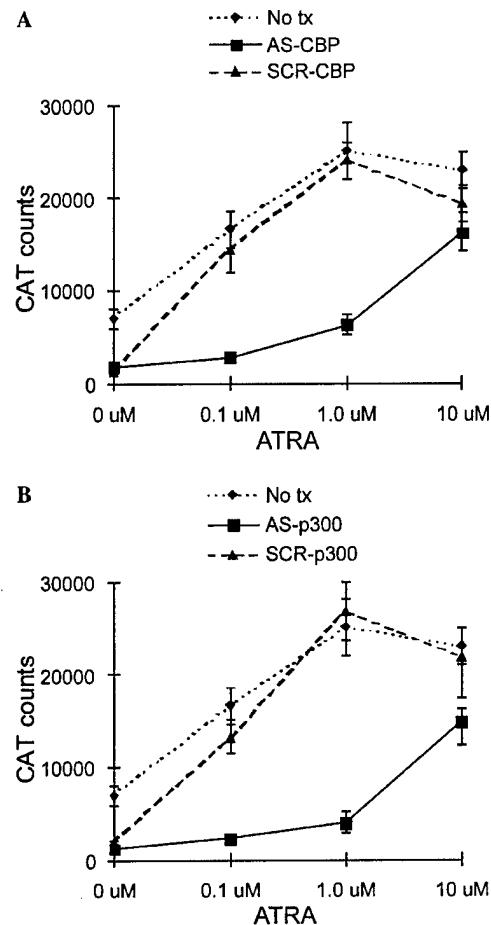
Fig. 2. HMECs treated with AS-ODNs show decreased trans-activation of a RARE-driven CAT reporter. (A) AS-CBP but not Scr-CBP decreases ATRA stimulated CAT activity and (B) AS-p300 but not Scr-p300 decreases ATRA stimulated CAT activity. HMECs were treated for 4 days with either AS-ODNs or Scr-ODNs, transfected with the RARE-CAT reporter pRRE4-tk CAT, treated with the indicated concentration of ATRA, and harvested, and the CAT activity was measured.

on CAT reporter activity at the same ATRA concentrations (Fig. 2B). Treatment of HMEC-SNs with Scr-CBP or -p300 did not alter ATRA stimulated CAT reporter expression (Fig. 2). The suppression of the RAR-β2 P2 promoter driven CAT reporter activity was largely overcome when cells were treated with 10 μM ATRA.

AS-CBP or -p300 treatment also blocked ATRA induced growth inhibition (data not shown). Also, AS-CBP or -p300 treatment decreased the alteration in the distribution of cells in G1- and S-phases in ATRA treated HMEC-SNs as compared to HMEC-SNs treated with Scr-ODNs to either CBP or p300 or to control HMEC-SNs (Fig. 3, Table 2). When pretreated with AS-ODNs, HMEC-SNs did not show a decreased growth rate when treated with either 0.1 or 1.0 μM
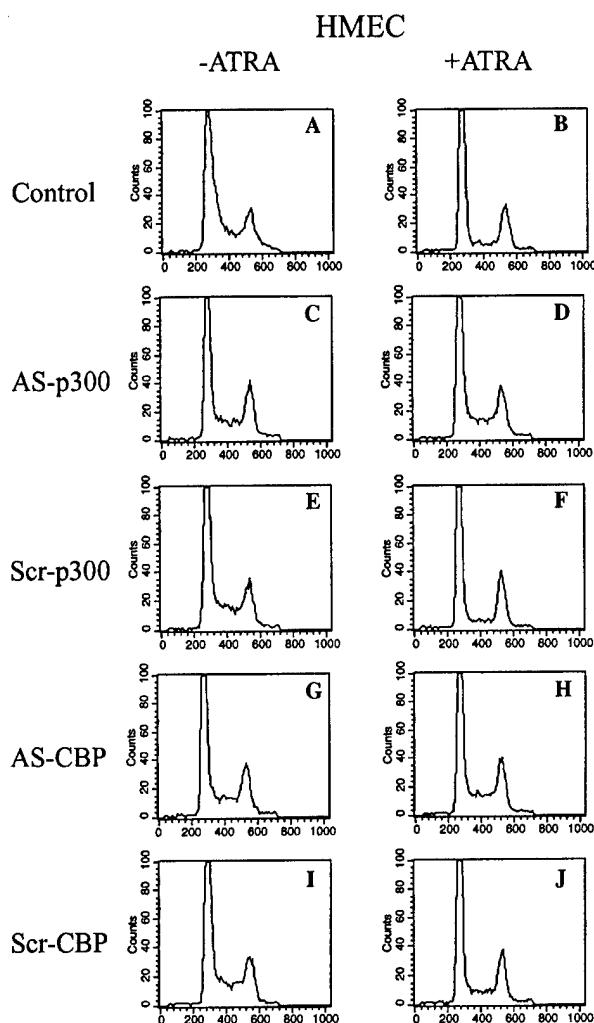
HMEC

Fig. 3. The effect of AS- and Scr-ODNs on the cell cycle distribution of HMEC-SN controls (A, C, E, G, and I) and after treatment with 1 μM ATRA for 4 days (B, D, F, H, and J). HMEC-SNs were pretreated with 1 μM AS- (C, D, G, and H) or Scr-ODNs (E, F, I, and J), harvested, stained with propidium iodide, and quantitated by FACS.

ATRA. Treatment with 10 μM ATRA, however, resulted in some growth inhibition. When pretreated with Scr-ODNs, HMEC-SNs treated with all ATRA concentrations showed growth inhibition similar to treated controls. HMEC-SNs treated with 1.0 μM ATRA, with or without Scr-CBP and -p300, showed a 50% decrease in the number of cells in S-phase and a 5–8% increase in the number of cells in G1-phase. HMEC-SNs pretreated with AS-CBP or AS-p300 showed a 10–20% decrease in S-phase cells and a 2–3% increase in G1-phase cells.

Discussion

We have previously shown that MCF7-β2s and HMEC-SNs are sensitive to ATRA while MCF7-SNs and HMEC-α403s are not [14,15]. These data demonstrate that both CBP and p300 mRNA and protein expression were induced by ATRA in MCF-7 and HMEC cells with functional retinoic acid signaling (Fig. 1, Table 1). Furthermore, the changes observed in mRNA levels in both MCF-7s and HMECs were directly reflected by changes in CBP and p300 protein levels. These data, however, do not exclude the possibility that CBP and p300 degradation is also regulated. Finally, AS-CBP or -p300 treatment could block ATRA induction of p300 and CBP, respectively, and ATRA induced trans-activation and growth suppression in HMECs (Tables 1C,D and 2, Figs. 2 and 3, data not shown).

The complimentary model systems used in this study were not fully equivalent. Neither CBP nor p300 protein levels were altered in MCF7-SNs when compared to MCF7-β2s in the absence of ATRA stimulation (Table 1A). In HMEC-α403s, however, there was a decrease in both CBP and p300 protein as compared to HMEC-SNs when no exogenous ATRA was added (Table 1B). Since the dominant negative α403 inhibits all RAR- and retinoid X receptor- (RXR) coupled signaling pathways [15], the observed dissimilarities between the systems were not unexpected. Inhibition of nonretinoid nuclear receptor signaling by RAR-α403 has been observed in other systems and was due to inhibition of signaling pathways in which a nonretinoid nuclear receptor heterodimerizes with RXR [29].

Table 2
Percentage distribution of cells in G_1- and S-phases after treatment with AS-CBP or AS-p300 followed by 1 μM ATRA for 4 days

Cell type	AS-ODN	1 μM ATRA	% G1	% ΔG1	% S	% ΔS
HMEC-SN	None	(−)	62		15	
		(+)	67	+8	7	−50
	AS-CBP	(−)	65		17	
		(+)	66	+2	15	−10
	Scr-CBP	(−)	61		15	
		(+)	64	+5	8	−50
	AS-p300	(−)	64		18	
		(+)	66	+3	15	−20
	Scr-p300	(−)	62		15	
		(+)	66	+6	8	−50

Data represents means of three experiments.

It has been previously shown that transcriptional regulation by ATRA is, in part, dependent on CBP and p300 [1,3,4]. The importance of CBP and p300 upregulation in the HMEC response to ATRA was again clearly demonstrated using AS-ODNs. CBP and p300 AS-ODNs were able to block upregulation of CBP and p300, respectively, and decrease both ATRA trans-activation of the RARE-driven CAT reporter by ATRA and suppression of HMEC growth (Figs. 2 and 3, data not shown, Table 2). AS-p300 and AS-CBP were not able to suppress ATRA induction of CBP and p300, respectively. This resulted in CBP and p300 levels that were comparable to untreated cells (Table 1C and D). After ATRA treatment, AS-CBP treated HMECs had decreased CBP and baseline p300 content and did not respond to ATRA. Similarly, AS-p300/ATRA treated HMECs had baseline CBP and decreased p300 content and also did not respond to ATRA. Interestingly, Scr-CBP/ATRA treated cells had increased levels of CBP and baseline levels of p300 and still responded normally to ATRA. Induction of p300 or CBP to higher than baseline levels was required to retain ATRA sensitivity.

Since CBP and p300 serve to integrate transcriptional regulation and the pool of CBP/p300 is limited, the ATRA associated increase of CBP/p300 pools in the cell might play an important role in global CBP/p300 co-factor regulated transcriptional control. Indeed, part of RAR-β2's role as a tumor suppressor could be attributable to upregulation of CBP and p300. Conversely, the loss of RAR-β2 expression might play a significant role in the downregulation of CBP and/or p300 in retinoid responsive tissues in vivo. Cells with low levels of CBP and/or p300 are likely to exhibit a poor response to chemoprevention or chemotherapeutic agents that depend on induction of either growth arrest or apoptosis. CBP and p300 levels are decreased in AML, treatment related CML and MDS, gastric cancer, and colorectal cancer as well as cell lines derived from lung, breast, and pancreatic cancers [28,30,31]. Therefore, induction of CBP and p300 could improve the response to such agents and result in increased efficacy of treatment. We are currently investigating whether increased expression of CBP or p300 in RAR-β2 negative cells increases their sensitivity to growth control and apoptotic signals to further characterize the relationship between RAR-β2 signaling and CBP/p300.

Acknowledgments

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CBP Modulates Reconstituted Extracellular Matrix-Growth Regulation, -Polarity, and -Apoptosis in Human Mammary Epithelial Cells

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ABSTRACT

Interactions between normal mammary epithelial cells (HMECs) and extracellular matrix (ECM) are important for mammary gland homeostasis; loss of ECM-sensitivity is thought to be an early event in mammary carcinogenesis. The CREBP binding protein (CBP) is known to regulate both proliferation and apoptosis but the role of CBP in ECM-signaling is poorly characterized. We investigated the relationship between CBP expression and sensitivity to prepared ECM (rECM)-induced growth arrest and apoptosis in an *in vitro* model of early mammary carcinogensis. Suppression of CBP expression in HMECs by antisense oligonucleotides (ODNs) resulted in loss of rECM-mediated growth regulation, polarity, and apoptosis. Chromatin immunoprecipitation studies (ChIP) and reporter studies demonstrated that inhibition of CBP protein expression resulted in 1) loss of CBP-occupancy of the *LAMA3A* promoter and 2) a decrease in *LAMA3A* promoter activity. rECM-resistance correlated with 1) loss of CBP occupancy of the *LAMA3A* promoter, 2) decreased *LAMA3A* promoter activity, and 3) loss of laminin-5 α3-chain mRNA and protein expression. These observations suggest a critical role for CBP in rECM-mediated growth regulation, polarity, and apoptosis through modulation of *LAMA3A* activity and laminin-5 α3-chain expression.

Introduction

Breast tissue is composed of mammary epithelial cells that rest on extracellular matrix (ECM)[1]. Interactions between epithelial cells and ECM regulate normal growth, polarity, and apoptosis (Folkman and Moscona, 1978; Petersen, et al. 1992; Strange, et al., 1992; Zutter, et al., 1995; Ilic et al., 1998; Farrelly, et al., 1999). Loss of ECM-signaling is thought to be an early event in mammary carcinogenesis and is postulated to promote some of the phenotypic changes observed during malignant progression (Petersen, et al., 1992; Howlett, et al., 1995; Farelly, et al., 1999; Mercurio, et al., 2001). Carcinogenesis is hypothesized to be a multistep process resulting from the progressive accumulation of genetic damage. While loss or mutation of specific tumor suppressor genes such as *TP53* promotes mammary carcinogenesis, not all damaged epithelial cells progress to malignancy and many are thought to be eliminated by apoptosis (Fabian, et al. 1996; Ashkenazi and Dixit, 1998; Rohan, et al. 1998). Mammary gland homeostasis requires a coordinated balanced between proliferation and programmed cell death. ECM-signaling is thought to play an important role in regulating this balance (Petersen, et al., 1992; Howlett, et al., 1995; Farelly, et al., 1999; Mercurio, et al., 2001). Loss of ECM-signaling is thought to result in apoptosis-resistance and may promote mammary carcinogenesis by preventing the apoptotic elimination of damaged mammary epithelial cells.

Laminins are ECM glycoproteins that promote mammary gland homeostasis through regulating cell adhesion, migration, proliferation, differentiation, and angiogenesis (Aberdam et al., 2000). Laminins have three distinct protein subunits, designated α, β, and γ. Laminin-5 (α3A, β3, and γ1) is the most abundant ECM glycoprotein produced by mammary epithelial cells (D'Ardenne, et al., 1991). Laminin-5 functions as a ligand for α3β1- and α6β4-integrins to regulate adhesion, migration, and morphogenesis (Stahl, et al., 1997). Breast cancers frequently demonstrate loss of laminin-5 expression and disruption of α3β1- and α6β4-integrins receptors, associated with loss of tissue organization, growth regulation, and polarity (D'Adenne, et al., 1991; Natali et al., 1992; Koukoulis, et al., 1993; Zutter, et al., 1995; Weaver, et al., 1999; Henning, et al. 1999; Shaw, 1999; Simpson-Haidaris and Rybarczyk, 2001; Seewaldt, et al., 2001*a*, Weaver, et al., 2002). Loss of laminin-5 α3- and γ2-chain expression is observed in malignant breast lesions while benign ductal and lobular epithelial cells demonstrate continuous laminin-5 staining at the

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epithelial-stromal interface (Henning, et al., 1999). These observations suggest that loss of laminin-5-signaling may be important for breast cancer progression.

We previously developed an *in vitro* model of early mammary carcinogenesis to investigate the potential role of ECM-signaling in eliminating acutely "damaged" HMECs (Seewaldt, et al., 2001*a*). Acute cellular damage was modeled by either 1) retroviral-mediated expression of the Human Papillomavirus Type-16 (HPV-16) E6 protein (HMEC-E6) or 2) treatment with p53-specific antisense oligonucleotides (ODNs) (p53(-) HMEC-AS). We observed that while HMEC controls grown in rECM underwent growth arrest on Day 7, HMEC-E6 and p53(-) HMEC-AS cells underwent apoptosis (Seewaldt, et al., 2001*a*). While the acute expression of either HPV-16 E6 or suppression of p53 in HMECs promoted sensitivity to rECM-mediated apoptosis, HMEC-E6 cells passaged in non-rECM culture rapidly acquired resistance to both rECM-mediated growth arrest and apoptosis associated with 1) loss of genetic material from chromosome 16 and 2) loss of polarized expression of the laminin-5 receptor, α3β1-integrin (Seewaldt, et al., 2001*a*). These observations lead us to hypothesize that 1) 16p might harbor a gene(s) whose loss and/or rearrangement may promote rECM-resistance and 2) laminin-5/α3β1-integrin-growth regulation and -polarity signals may be critical for targeting the elimination of acutely "damaged" HMECs.

In these studies, HPV-16 E6 was expressed in HMECs as a model of acute cellular damage. E6 interacts with a large number of cellular proteins critical for normal growth regulation and apoptosis (O'Connor, 2000). HPV-16 E6 binds to p53 and targets it for ubiquitin-mediated degradation and also abrogates p53 transcriptional activity (Zimmermann, et al., 1999; O'Connor, 2000; zur Hausen, 2000). E6 has been shown to interact with the CREBP-binding protein, CBP, at the C/H1 and C/H3 domains as well as sequences near the carboxy-terminus (O'Connor, 2000; Patel, et al. 1999). HPV-16 E6 protein also activates telomerase (Klingelhurtz, et al., 1996), binds to Bak (Thomas and Banks, 1998), physically interacts with the focal adhesion proteins, paxillin and fibulin-1 (Tong and Howley, 1997), and interacts with the transcriptional regulator, interferon regulatory factor-3 (Ronco, et al. 1998). Taken together, these observations provide evidence that expression of HPV-16 E6 in HMECs leads to marked cellular damage.

Detailed cytogenetic analysis performed in this report indicate that chromosome 16p13 is the critical area whose loss and/or rearrangement promotes rECM-resistance. CBP, is a nuclear protein located at chromosome band 16p13.3 that regulates proliferation, differentiation, and apoptosis (Yao, et al., 1998; Giles, et al., 1997*a*). CBP is a key integrator of diverse signaling pathways including those regulated by retinoids, p53, estrogen, and BRCA1 (Kawasaki, et al., 1998; Robyr, et al., 2000). Chromosomal loss at 16p13 has been reported to occur in a majority of benign and malignant papillary neoplasms of the breast and loss or amplification of 16p is frequently observed in premalignant breast lesions (Lininger, et al., 1998; Tsuda, et al., 1998; Aubele, et al., 2000). Taken together, these observations suggest that loss of CBP expression might promote mammary carcinogenesis.

Prior studies indicated that laminin-5/α3β1-integrin-growth regulation and -polarity signals might be critical for targeting the elimination of acutely damaged HMECs (Seewaldt et al., 2001*a*). Little is known about the regulation of laminin-5 gene transcription in normal mammary epithelial tissue, nor about the molecular mechanism underlying the loss of laminin-5 expression observed in early breast carcinogenesis (Miller, et al., 2000). The human *LAMA3A* promoter is known to contain three binding sites of the dimeric transcription factor, activating protein-1 (AP-1) (Virolle, et al., 1997; Miller, et al., 2001). It has been recently observed that the second AP-1 binding site present in the human *LAMA3A* promoter at position –185 base pairs is critical for baseline transcription of laminin-5 α3-chain (Miller, et al., 2001). CBP is known to interact with AP-1 response elements (Benkoussa, et al., 2002). However, the relationship between CBP and laminin-5 expression in mammary epithelial cells has not been studied.

This report describes a novel role for CBP in mediating sensitivity to rECM-growth regulation, -polarity, and –apoptosis through induction of laminin-5 α3-chain expression. Observations in our model system have important implications as they predict a critical role for CBP in regulating mammary homeostasis and targeting the elimination of acutely "damaged" HMECs through a laminin-5 α3-signaling pathway.

Materials and Methods

Cell Culture and Media

Normal human mammary epithelial cell (HMEC) strains AG11132 and AG1134 (M. Stampfer #172R/AA7 and #48R, respectively) were purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute) (Stampfer, 1985). HMEC strains AG11132 and AG11134 were established from normal tissue obtained at reduction mammoplasty, have a limited life span in culture, and fail to divide after approximately 20 to 25 passages. HMECs exhibit a low level of estrogen receptor staining characteristic of normal mammary epithelial cells. HMECs were grown in Mammary Epithelial Cell Basal Medium (Clonetics, San Diego, CA) supplemented with 4 µl/ml bovine pituitary extract (Clonetics #CC4009), 5 µg/ml insulin (Sigma, St. Louis, MO), 10 ng/ml epidermal growth factor (UBI Lake Placid, NY), 0.5 µg/ml hydrocortisone (Sigma), 10^{-5}M isoproterenol (Sigma), and 10 mM HEPES buffer (Sigma) [Standard Media]. Cells were cultured at 37°C in a humidified incubator with 5% CO_2/95% air. Mycoplasma testing was performed as previously reported (Seewaldt et al., 1997a).

Retroviral Transduction

The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence was provided by D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) (Demers et al., 1996). HMECs (passage 8) were plated in four T-75 tissue culture flasks (Corning, Corning, NY) in Standard Medium and grown to 50% confluency. Transducing virions from either the PA317-LXSN16E6 or the control PA317-LXSN (without insert) retroviral producer line were added at a multiplicity of infection at 1:1 in the presence of 4 µg/ml Polybrene (Sigma) to log-phase cells grown in T-75 flasks (Seewaldt, et al., 1995). The two remaining T-75 flasks were not infected with virus. After 48 hours, two flasks containing transduced cells and one flask with untransduced cells were passaged 1:3 (passage 9) and selected with Standard Media containing 300 µg/ml G418. Cells were grown in Standard

Media containing 300 µg/ml G418 (Gibco, Grand Island, NY) for four to seven days, until 100% of control, untransduced cells were dead. Cells were passaged 1:3 at the completion of selection (passage 10), and were maintained in the absence of selection before immediately proceeding to apoptosis experiments. The fourth flask of unselected, untransduced parental control cells was passaged in parallel with the selected, transduced experimental and vector control cells. Parental AG11132 cells were designated HMEC-P. Transduced AG11132 cells expressing the HPV-16 E6 construct were designated HMEC-E6 and vector control clones were designated HMEC-LXSN. All cells were maintained in Standard Media after transfection in the absence of G418 selection to ensure that any observed chromosomal abnormalities or apoptosis-resistance was not due to continued exposure to G418. All experiments were performed on mass cultures. Mycoplasm testing was as previously reported (Seewaldt, et al., 1997*b*).

Cytogenetic Analysis of Early and Late Passage HMECs

Spectral analyses (SKY) of HMEC-LXSN controls (passages 10 and 16) and HMEC-E6 cells (passages 10 and 18) were performed as previously described (Mrózek, et al., 1993; Schröck, et al., 1996; Seewaldt, et al., 2001*a, b*).

Western Blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt, et al. 1997*b;* Seewaldt, et al. 1999*b*). For p53 expression, the membrane was incubated with a 1:100 dilution of mouse anti-human p53 (Oncogene Science Ab-2). For CBP expression, the blocked membrane was incubated with 1:200 dilution of the CBP C20 antibody (Santa Cruz Biotechnology). For laminin 5 expression the membrane was incubated with a 1:100 dilution of the C-19 antibody to the laminin-5 α3-chain (Santa Cruz). Loading control was provided by 1:200 dilution of the I-19 antibody to beta-actin (Santa Cruz). The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software (Kodak, Rochester, NY).

HMEC Culture in rECM

HMECs were grown in rECM as previously described (Seewaldt et al., 2001*a*). 100 μl of rECM (Growth Factor Depleted Matrigel™, Collaborative Research, Bedford, MA) were added per well to a 48 well plate and allowed to gel at 37°C for 20 min. Transduced HMECs were trypsinized, counted, and pelleted in a sterile microcentrifuge tube. Approximately 1 x 10⁴ cells were resuspended in 100 μl rECM on ice, gently overlaid on the initial undercoating of extracellular matrix, and allowed to gel at 37° C for 20 min. Standard Media was then added and wells were inspected to ensure there was an equal distribution of cells in each well. Cells were grown for 5 to 13 days in culture.

Cell Growth and Proliferation in rECM Culture

Cell growth in rECM culture was determined by the following criteria: the size of growing spherical cell colonies was measured with an eye-piece equipped with a micrometer spindle. The 20 largest colonies were measured. Proliferation was assessed by Ki67 staining index as follows: five micron sections were immunostained with antibody directed against Ki-67. Cells were scored visually (100-500 cells) for immunopositive nuclei. The proliferation index was calculated by dividing the number of immunopositive cells as a percentage of the total number of cells scored.

Detection of Apoptosis by In Situ TUNEL

HMECs were grown in rECM for 5 to 11 days and prepared for TUNEL staining as previously described (Seewaldt, et al., 2001*a*). Two hundred cells were scored. The apoptotic index was determined by expressing the number of TUNEL positive cells as a percentage of the total number of cells scored.

Transmission Electron Microscopy

HMECs were grown in contact with rECM as described above. Electron microscopy was

as previously described (Seewaldt, et al., 1999*a*; Dietze, et al., 2001). Fifty colonies were scored for the presence of apoptosis by morphologic criteria that included 1) margination of chromatin, 2) nuclear condensation, 3) cell shrinkage, and 4) formation of apoptotic bodies (Majno and Joris, 1995).

Immunostaining

HMECs were grown in rECM as described above and embedded in O.C.T. (Miles), snap frozen, and 7 μ sections obtained. For E-cadherin staining: sections were fixed with 3.7% formaldehyde in PBS for 30 min at RT and were blocked with 0.5% heat-denatured bovine serum albumin (HD-BSA) in PBS for 1 hr at RT. Sections were then incubated for 30 min with mouse anti-human E-cadherin antibody (BD Signal Transduction Laboratories) diluted in PBS with 0.5% HD-BSA for 30 min at RT and then washed 6 times with PBS at RT. For integin and laminin immunostaining: cells were fixed for 20 min at RT with 2% formaldehyde in 0.1 M sodium cacodylate, and 0.1 M sucrose at pH 7.2, permeabilized with 0.1% Triton X-100 for 10 min at RT, and blocked with 0.5% HD-BSA in PBS for 1 hr at RT. Cells were incubated with a primary antibody diluted in PBS with 0.5% HD-BSA for 1 hr at RT and washed 6 times with PBS at RT. Antibodies against integrin subunits α3 (P1F2, P1B5), and β1 (P4C10) were a generous gift of William Carter and have been previously described (Carter, et al., 1990*a, b*; Wayner and Carter, 1987; Wayner, et al., 1988). Monoclonal antibodies P5H10 directed against the α3 chain of laminin-5 were a generous gift of William Carter. For immunoflurorescence, cells were incubated with either FITC- or Rhodamine-conjugated goat anti-mouse antibody at a 1:200 antibody dilution (Santa Cruz) in PBS with 0.5% HD-BSA for 30 min at RT and washed. Sections were mounted in 30% glycerol in PBS and visualized for immunofluorescence using a Zeiss LSM 410 fluorescence microscope (Carl Zeiss, Jena, Germany).

Suppression of CBP Expression

Nine antisense oligonucleotides (ODNs) to human CBP were generated by the PAS

program (Ugai, et al., 1999). The CBP antisense A3342V ODN (24-mer, nucleotide position 3342-3363) was initially chosen on the basis of selective inhibition of CBP protein expression in MCF-7 cells (data not shown); suppression was confirmed in HMECs. Inactive CBP ODN A2172Z (26 mer, nucleotide position 2172-2197) was 1) chosen to be the scrambled sequence of the antisense ODNs to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content and 2) selected based on lack of suppression of CBP in MCF-7 and HMECs. See Table 1 for a list of ODNs. The first and last three nucleotides of all ODNs were phosphorothioate modified to increase their stability *in vitro*. Early passage HMEC-LXSN controls and HMEC-E6 cells were plated in T-75 plates in Standard Media. After allowing 24 hr for attachment, cell cultures were treated for 72 hr with either active or inactive ODNs (0.001 to 0.1μM final concentration). Every 24 hr the culture media was replaced by new Standard Media containing fresh ODNs. Western analysis was performed to confirm 1) suppression of CBP expression as described above and 2) lack of suppression of the related co-activator p300. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software.

CBP-Suppression in rECM Culture

Early passage HMEC-LXSN controls and HMEC-E6 cells were trypsinized and approximately 1×10^4 cells were resuspended in 100 μl rECM containing either active or inactive CBP-specific ODNs (0.01 to 0.1 μM final concentration) on ice. rECM cultures were prepared as above. rECM cultures were overlayed with Standard Media containing active or inactive CBP-specific ODNs (0.01 to 0.1 μM final concentration). Overlay media were changed every 24 hours to ensure a fresh supply of ODNs. The diameter of the growing colonies was determined and cells were prepared for electron microscopy and immunostaining as described above.

Large-Scale rECM Culture

Large-scale rECM culture was utilized to prepare total RNA or protein lysate for analysis utilizing techniques previously developed by the laboratory of Minna Bissell (Roskelly, et al., 1994). Early and late passage HMEC-E6 cells and HMEC-LXSN controls were plated in T-75 flasks, previously treated with poly(2-hydroxyethyl methacrylate) (Poly-HEME). Cells were grown in Standard Media with 5% (v:v) rECM.

Differential Gene Expression Studies

Cells were grown in rECM utilizing large-scale rECM culture techniques as described above. Isolation of total RNA was as previously described (Seewaldt, et al., 1995). RNA integrity was confirmed by electrophoresis, and samples were stored at −80°C until used. All RNA combinations used for array analysis were obtained from cells that were matched for passage number, cultured under the identical growth conditions, and harvested at identical confluency. cDNA synthesis and probe generation for cDNA array hybridization were obtained by following the standardized protocols provided by Affymetrix™ (Affymetrix, Santa Clara, California).

Expression data for approximately 5,600 full-length human genes was collected using Affymetrix GeneChip HuGeneFL™ arrays, following the standardized protocols provided by the manufacturer. Data was collected in triplicate using independent biological replicates (Baldi and Long, 2001). Array images were processed using Affymetrix MAS 5.0 software, where we filtered for probe saturation, employed a global array scaling target intensity of 1000, and collected the signal intensity value for each gene. For each set of replicate measurements, gene signal intensity values were averaged and an array-level normalization was performed relative to the β-actin averaged value. Normalized values were $\log_2$ transformed and an additional gene-level normalization was performed using the LXSN-ECM data set. Data was imported into *Cluster/TreeView* software (http://rana.lbl.gov/EisenSoftware.htm), where heat maps were generated.

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Semi-Quantitative RT-PCR

To confirm microarray data, relative transcript levels were analyzed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Five micrograms of total RNA was used in first-strand cDNA synthesis with Superscript II reverse transcriptase (Invitrogen). PCR reaction conditions were optimized for integrin-α3 (*ITGA3*), integrin-β1 (*ITGB1*), laminin-α3 (*LAMA3*), laminin-β3 (*LAMB3*), and laminin-γ2 (*LAMC2*). Primer sequences were obtained from published sources as follows: ITGA3 (Hashida, et al., 2002), ITGB1 (Hsu, et al., 2001), LAMA3 (forward primer, Virolle, et al., 2002), LAMB3 and LAMC2 (Manda, et al., 2000). A 50 μl reaction was set up containing 100 nM forward primer, 100 nM reverse primer, 250 μM of each dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, 2.5 units Taq polymerase, and 2.0 μl cDNA. Reaction conditions for beta-actin were 300 nM forward primer, 300 nM reverse primer, 250 μM of each dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH = 8.3, 2.5 units Taq polymerase, and 2.0 μl cDNA in a total volume of 50 μl. Products were amplified with Perkin Elmer GeneAmp PCR system 2400. Preliminary reactions were preformed to determine the PCR cycle number of linear amplification for each primer set. The primer sets, cycling conditions, and cycle numbers used are indicated in Table 2. Ten microliters of PCR product were analyzed by electrophoresis in 1.2-1.5% agarose gels containing ethidium bromide and visualized under UV light and quantitated. All samples were performed in triplicate and normalized to beta-actin control.

LAMA3A Reporter Studies

A 1403 bp region of the laminin-5 α3 (*LAMA3*) promoter corresponding to GenBank Accession Number AF279435 was amplified with PCR primers, sense 5'-AAG CTT AAG TTT TCC CAT CCG CAA C-3' and antisense 5'-TCT AGA GCT GAC CGC CTC ACT GC-3' (Miller, et al., 2001). The PCR product was cloned into pCRII

(Invitrogen), digested out with HindIII and BamHI, and cloned into the reporter plasmid pBLCAT5 (ATCC). Cells were transfected with the resultant pBLLAMA3aCAT5 reporter plasmid using previously published transfection conditions and controls (Seewaldt et al., 1997*a*). Transfected cells were plated in Standard Media in T-25 flasks pre-treated with Poly-HEME, treated with 5% (v:v) rECM for 24 hr, and harvested for CAT activity assays as previously described (Seewaldt, et al. 1997*a*).

Occupancy of the AP-1-"rich" region of *LAMA3A* promoter from positions –387 to –127 was tested by chromatin immunopreciptiation (ChIP). ChIP was performed by published methods with some modifications (Yahata, et al., 2001). Early and late passage HMEC-E6 cells and HMEC-LXSN controls were plated in T-25 flasks treated with Poly-HEME and grown in Standard Media with 5% (v:v) rECM. Preliminary experiments were run to determine optimal sonication and formaldehyde cross-linking time. Once optimized, cells were harvested, pelleted, and treated with 1% formaldehyde for 15-20 minutes to cross-link cellular proteins. The formaldehyde was quenched by adding 1.0 ml of 250 mM glycine followed by a 5 min RT incubation. Cells were then rinsed twice in ice cold PBS containing protease inhibitors, pelleted, and resuspended in Lysis Buffer [1% SDS, 10 mM EDTA, 50mM Tris-HCl at pH 8.1, 1x Protease Inhibitor Cocktail (4 µg/ml epibestatin hydrochloride, 2 µg/ml calpain inhibitor II, 2 µg/ml pepstatin A, 4 µg/ml mastoparan, 4 ug/ml leupeptin hydrochloride, 4 µg/ml aprotinin, 1 mM TPCK, 1 mM phenylmethylsulfonyl fluoride, and 100 µM TLCK)]. Samples were then sonicated 3 x 15 seconds each with a 1 min incubation on ice in between pulses on a Branson sonifier model 250 at 50% duty and maximum mini probe power. Supernatants were diluted (1:10) in Dilution Buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1, 1x Protease Inhibitor Cocktail], and precleared with 2 µg of sheared salmon sperm DNA, 20 µl normal human serum, and 45 µl of protein A-sepharose [50% slurry in 10 mM

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Tris-HCl at pH 8.1, 1 mM EDTA]. Human anti-CBP antibody (A22, Santa Cruz) was added to the precleared lysate, and placed on a shaker at 4 ^{0}C, followed by the addition of 45 µl of protein A-sepharose and 2.0 µg sheared salmon sperm DNA, and an additional 1 hour incubation on a shaker at 4 ^{0}C. Sepharose beads were then collected and washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), and buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1). Beads were washed once with TE buffer and DNA eluted with 100 µl of 1% SDS-0.1 M NaHCO$_3$. Eluate was heated at 65°C overnight to reverse the formaldehyde cross-linking. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation, and then amplified by using PCR primers, sense 5'-AAG CTT AAG TTT TCC CAT CCG CAA C-3' and antisense 5'-TCT AGA GCT GAC CGC CTC ACT GC-3'. Thirty microliters of PCR product were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized under UV light. All samples were performed in triplicate.

Results

Late passage HMEC-E6 cells acquire resistance to rECM-induced apoptosis associated with rearrangement of the CBP locus at chromosome 16p13.

Previous cytogenetic analysis suggested that chromosome 16p harbored a gene(s) whose loss and/or rearrangement might play a role in resistance to rECM-mediated growth control and apoptosis (Seewaldt, et al. 2001*a*). In this study, detailed SKY-based cytogenetic analysis was performed on rECM-resistant, late passage HMEC-E6 cells to identify gene rearrangements that might pinpoint the chromosomal location of the gene of interest. Chromosomal rearrangements and deletions involving 16p were surveyed in 35 unique late passage HMEC-E6 cells. A majority of chromosomal changes involving 16p were whole chromosome or whole chromosome arm deletions, however, 1) one rECM-resistant cell with a 16p deletion retained material proximal to 16p12 while, 2) a second rECM-resistant cell exhibited an unbalanced translocation der(16)t(13;16)(q1?2;p13) that affected band 16p13 (Figure 1). These observations indicated that the gene of importance was located in the distal region of 16p, at 16p13. Chromosomal band 16p13 is the locus of the CBP gene (Giles, et al., 1997*a,b*; Yao, et al., 1998). Since CBP is known to play a role in growth regulation and apoptotic signaling, we hypothesized that loss of CBP protein expression might promote rECM-resistance in HMEC-E6 cells.

Resistance to rECM-mediated growth regulation and apoptosis correlates with a decrease in CBP protein expression.

Western analysis tested whether rECM-resistant late passage HMEC-E6 cells exhibited decreased expression of CBP. CBP protein expression was markedly decreased in late passage, rECM-resistant HMEC-E6 cells relative to early passage, rECM-sensitive HMEC-E6 cells and HMEC-LXSN controls. Late passage HMEC-E6 cells exhibited a 41% ($p \leq$ 0.01) and 72% ($p \leq$ 0.01) respective decrease in CBP protein expression relative to early passage HMEC-LXSN controls and early passage HMEC-E6 cells (Figure 2 *c*). In contrast, there was no significant decrease in CBP protein expression in late passage

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HMEC-LXSN controls relative to early passage cells (Figure 2 *c*). These observations in late passage HMEC-E6 cells 1) demonstrate that rECM-resistance correlates with decreased CBP protein expression and 2) are consistent with cytogenetic analysis demonstrating loss or rearrangement of the CBP locus at 16p13.

Suppression of CBP in early passage HMECs by antisense ODNs.

Antisense ODNs were utilized to suppress CBP protein expression in HMECs to test whether the level of CBP protein expression might be important for rECM-sensitivity. Relative levels of CBP protein expression were tested by Western analysis. Early passage HMEC-LXSN control and early passage HMEC-E6 cells treated with the active, CBP-specific ODN, A3342V, exhibited a 65% and 72% respective decrease in CBP protein expression relative to untreated controls (Figure 2 *d*). Cells treated with the inactive CBP ODN, scrA3342V, did not exhibit a significant decrease in CBP protein expression (Figure 2 *d*).

Suppression of CBP enhances proliferation in rECM.

Treatment of early passage HMEC-LXSN controls and HMEC-E6 cells with CBP-specific ODNs resulted in enhanced proliferation in rECM-culture as measured by 1) physical growth parameters and 2) Ki-67 staining. Both early passage HMEC-LXSN controls and HMEC-E6 cells treated with active CBP-specific ODNs (A33243V) demonstrated a continued increase in sphere diameter from Day 7-9 in rECM culture (Figure 3 *a, b*). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive ODNs (scrA33243V) did not exhibit an increase in sphere diameter after Day 7 (Figure 3 *a, b*). Treatment of early passage HMEC-LXSN and HMEC-E6 cells with CBP-specific ODNs (A33243V) resulted in continued Ki-67 staining at 9 and 11 days in rECM culture (Figure 3 *c, d*). In contrast, Ki-67 staining at 9 and 11 days were markedly reduced in HMEC-LXSN and HMEC-E6 cells treated with inactive ODNs (scrA33423V) (Figure 3 *c, d*). These observations show that suppression of CBP protein expression in early passage

HMEC-E6 cells and HMEC-LXSN controls resulted in enhanced proliferation in rECM culture.

Suppression of CBP protein results in altered localization and expression of biochemical markers of polarity.

Early passage HMEC-E6 cells and HMEC-LXSN controls treated with active CBP ODNs (A33423V) exhibited a loss of epithelial polarity as evidenced by dispersed and intracellular staining of 1) E-cadherin and 2) the tight junction-associated protein, ZO-1 (Figure 4). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODNs (scrA33423V) demonstrated expression of E-cadherin and ZO-1 at the cell-cell junction, consistent with a correctly polarized epithelium (Figure 4). These observations indicate that suppression of CBP protein expression in HMECs by antisense ODNs promotes a loss of epithelial polarity in rECM culture.

Suppression of CBP inhibits apoptosis in rECM culture.

Early passage HMEC-E6 cells were treated with CBP-specific antisense ODNs to test whether suppression of CBP protein expression blocked apoptosis in rECM culture. Early passage HMEC-E6 treated with CBP-specific antisense ODNs (A33423V) formed large irregular clusters in rECM and did not undergo apoptosis as assessed by either electron microscopy or TUNEL-staining (Figure 5 *b, d*). In contrast, early passage HMEC-E6 cells treated with inactive CBP ODNs underwent apoptosis on Day 7 as assessed by either morphologic criteria or TUNEL-staining (Figure 5 *c, d*). Similar to early passage HMEC-E6 cells, early passage HMEC-LXSN cells treated CBP-specific, antisense ODNs (A33423V) formed large irregular clusters in rECM (Figure 5 *a*). Early passage HMEC-LXSN controls treated with inactive ODN (scrA33423V) formed a morphologically organized, ascinus-like structure and did not undergo apoptosis consistent with what has been previously observed for early passage HMEC-LXSN untreated controls (Figure 5 *e* and data not shown) (Seewaldt, et al., 2001*a*). These observations demonstrate that

suppression of CBP protein expression in early passage HMEC-E6 cells by antisense

ODNs blocks apoptosis in rECM culture.

A second HMEC strain, AG11134, was tested to ensure that these observations were not

HMEC strain-specific. Similar to observations made in HMEC strain AG11132 above, 1)

early passage AG11134-E6 cells treated with inactive CBP ODN (scrA33423V) were

sensitive to rECM-growth regulation and underwent apoptosis at Day 7 (data not shown), 2)

early passage AG1134-LXSN controls treated with antisense-CBP ODN (A99424V) were

resistant to rECM growth arrest and did not undergo apoptosis at Day 7-9 (data not shown),

and 3) early passage AG11134-LXSN controls treated with CBP-specific antisense ODNs

were resistant to rECM-mediated growth regulation and did not undergo apoptosis (data not

shown).

Laminin-5 expression is decreased in rECM resistant, late passage HMEC-E6 cells.

We previously observed that sensitivity to rECM-apoptosis in early passage HMEC-E6

cells required polarized expression of $\alpha 3/\beta 1$-integrin (Seewaldt et al., 2001*a*). Differential

gene expression studies, semi-quantitative RT-PCR, and Western analysis were performed

to test whether the loss of sensitivity to rECM-mediated growth regulation, polarity, and

apoptosis observed in late passage HMEC-E6 cells correlated with altered expression of

laminin-5 and/or $\alpha 3/\beta 1$-integrin mRNA. Differential gene expression studies demonstrated

decreased expression of all three laminin-5 chains ($\alpha 3$, $\beta 3$, and $\gamma 2$) in apoptosis-resistant,

late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls and early

passage HMEC-E6 cells grown in rECM (Figure 6 *a*). Semi-quantitative RT-PCR

confirmed a 98% decrease in laminin-5 $\alpha 3$-chain (p$\leq$0.01), an 88% decrease in laminin-5

$\beta 3$-chain (p$\leq$0.01), and a 75% decrease in laminin-5 $\gamma 2$-chain (p$\leq$0.01) mRNA expression

relative to early passage HMEC-LXSN controls (Figure 6 *b, c*). There was no significant

change in the level of $\alpha 3/\beta 1$-integrin mRNA expression (Figure 6 *a, b, c*). Western

analysis similarly demonstrated an 85% (p<0.001) decrease in laminin-5 α3-chain protein expression in apoptosis-resistant, late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls (Figure 6 *d*). Expression of laminin-5 α3-chain protein did not significantly vary between early passage HMEC-LXSN cells and early passage HMEC-E6 cells (Figure 6 *d*). There was a 130% (p<0.002) increase in laminin-5 α3-chain protein in late passage HMEC-LXSN controls relative to early passage HMEC-LXSN cells. These observations demonstrate that the presence of rECM-resistance in late passage HMEC-E6 cells correlates with a loss of laminin-5 mRNA and protein expression.

Lack of polarized expression of laminin-α3 and integrin-α3 proteins in late passage HMEC-E6 cells grown in rECM.

Early and late passage HMEC-LXSN controls and HMEC-E6 cells were grown in rECM and tested for 1) laminin-5 α3-chain and 2) α3- and β1-integrin expression by immunohistochemistry (clones P5H10, P1F2, and P4C10, respectively). Early and late passage HMEC-LXSN controls and early passage HMEC-E6 cells exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins (Figure 7 *a-i*). In contrast, late passage, CBP-"poor" HMEC-E6 cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of both laminin-5 α3-chain and α3-integrin (Figure 7 *j, l*). As predicted by differential gene expression studies and Western analysis, there was also a qualitative decrease in laminin-5 α3-chain expression in late passage HMEC-E6 cells relative to controls (Figure 7 *l*). Late passage HMEC-E6 cells grown in rECM exhibited polarized basal β1-integrin expression but had an increase in the amount of cytosolic expression relative to early passage cells (Figure 7 *k*). These observations demonstrate a loss of polarized expression of laminin-5 α3-chain and α3-integrin in rECM-resistant late passage HMEC-E6 cells.

Suppression of CBP expression in HMECs alters both laminin-α3 and integrin-α3

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protein expression in rECM culture.

We observed that late passage HMEC-E6 cells grown in rECM culture exhibit 1) reduced levels of CBP protein expression and 2) disorganized expression of both laminin-5 α3-chain and α3-integrin. This observation led us to hypothesize that suppression of CBP in HMECs would alter laminin-5 α3-chain and α3-integrin expression and/or distribution. CBP protein expression was suppressed in early passage HMEC-E6 cells and HMEC-LXSN controls by treatment with CBP-specific, antisense ODN (A99424V). HMECs with suppressed CBP expression exhibited disorganized plasma membrane and cytosolic distribution of laminin-5 α3-chain and α3-integrin (Figure 8 *a, b, i, j*). β1-integrin expression was observed at the basal surface (Figure 8 *e, f*). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODN (scrA99424V) exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins (Figure 8 *c, d, g, h, k, l*). These observations demonstrate that suppression of CBP protein expression in HMECs alters the distribution of both laminin-5 α3-chain and α3-integrin.

Decreased CBP expression in rECM-resistant late passage HMEC-E6 cells correlates with decreased LAMA3A promoter activity.

We tested whether the observed decrease in CBP and laminin-5 α3-chain expression in late passage HMEC-E6 cells correlated with decreased *LAMA3A* promoter activity. Early and late passage HMEC-E6 and passage-matched HMEC-LXSN controls were transiently transfected with a CAT reporter coupled to the *LAMA3A* promoter sequence (1403 bp, GenBank Accession Number AF279435) and grown in rECM culture. rECM-sensitive early passage HMEC-E6 cells and early and late passage HMEC-LXSN controls exhibited a similar level of *LAMA3A* activity (Figure 9 *a*). In contrast, rECM-resistant, late passage HMEC-E6 cells with decreased CBP and laminin-5 α3-chain expression exhibited a 91% decrease in *LAMA3A* promoter activity relative to early passage HMEC-E6 cells (p≤0.01) (Figure 9 *a*). These experiments demonstrate in HMECs a positive correlation between 1)

the level of CBP and laminin-5 α3-chain protein expression and 2) *LAMA3A* promoter activity.

LAMA3A promoter activity in HMECs with suppressed CBP protein expression.

We next tested whether suppression of CBP protein expression resulted in decreased *LAMA3A* promoter activity. *LAMA3A*-CAT reporter activity was compared in early passage HMEC-LXSN and HMEC-E6 cells treated with either CBP-specific antisense ODNs (A33423V) or inactive ODNs (scrA33423V). A 92% and 89% decrease (p<0.01) in *LAMA3A* promoter activity was observed, respectively, in early passage HMEC-LXSN and HMEC-E6 cells grown in rECM and treated with CBP-specific ODNs (A33423V) relative to cells treated with inactive ODNs (scrA33423V) (Figure 9 *b*). No significant difference in *LAMA3A* promoter activity was observed in HMECs treated with or without inactive ODNs. These observations demonstrate that suppression of CBP expression in HMECs results in a reduction in *LAMA3A* promoter activity.

Lack of CBP occupancy of the LAMA3A promoter correlates with rECM-resistance.

The human *LAMA3A* promoter contains three AP-1 sites at positions −387, −185, and −127 (Miller et al., 2001). The AP-1 site, at position −185, has been previously shown to be critical for basal activity in mammary epithelial cells (Miller, et al., 2001). Chromatin immunopreciptation (ChIP) was performed in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells and controls to test whether the observed 1) decrease in laminin-5 α3-chain expression and 2) loss of *LAMA3A* activity correlated with a lack of CBP binding to the 277 bp AP-1-"rich" site of the *LAMA3A* promoter (position -402 to −125) (Miller, et al., 2001). Early and late passage HMEC-LXSN control cells and rECM-sensitive, early passage HMEC-E6 cells grown in rECM demonstrated CBP binding to the AP-1-"rich" site of the *LAMA3A* promoter. In contrast, rECM-resistant, late passage HMEC-E6 cells, with decreased CBP and laminin-5 α3-chain expression, failed to demonstrate CBP binding

(Figure 10a). These observations suggest that a decrease in CBP expression might promote loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter.

Suppression of CBP expression in HMECs results in loss of CBP occupancy of the LAMA3A promoter.

Early passage HMEC-E6 cells were treated with active CBP ODNs and tested by ChIP to determine whether suppression of CBP protein expression resulted a loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Early passage HMEC-E6 cells treated with CBP-specific ODNs, and grown in rECM, did not demonstrate CBP occupancy of the *LAMA3A* promoter (Figure 10 b). In contrast, early passage HMEC-E6 controls, treated with inactive ODNs, and grown in rECM demonstrated CBP-occupancy (Figure 10 b). These observations demonstrate that suppression of CBP expression in HMEC-E6 cells by antisense ODNs results in a loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter. Since the AP-1 site, at position –185, is critical for basal activity in mammary epithelial cells (Miller, et al., 2001), these observations provide a mechanism by which loss of CBP expression might promote loss of *LAMA3A* promoter activity and laminin-5 α3-chain expression in HMECs.

Discussion

ECM has been shown to provide signals critical for mammary epithelial cell survival; in their absence cells undergo apoptosis (Streuli, et al., 1991; Strange, et al., 1992; Pullan, et al., 1996; Ilic, et al., 1998; Boudreau, et al., 1998; Bissell, et al., 1999). However, there is also evidence that growth arrest and survival signals promote apoptosis in genetically damaged cells (Seewaldt, et al., 1995; Wahl, et al., 1996; Hong and Sporn, 1997; Mancini, et al., 1997; Seewaldt, et al., 1997*b*; Seewaldt et al. 2001*a*). We previously demonstrated that rECM-derived growth regulation and polarity signals promoted apoptosis in our model of early breast carcinogenesis. Based on these observations we hypothesized that resistance to rECM-regulated growth arrest and polarity would promote apoptosis-resistance.

In this report we show that partial suppression of CBP protein expression in HMECs 1) results in loss of growth regulation and polarity in rECM culture and 2) blocks apoptosis in acutely damaged HMEC-E6 cells. This is the first demonstration that CBP-regulated growth and polarity signaling may be important for regulating apoptosis in "acutely damaged" HMECs. CBP is a tightly regulated transcription factor that regulates proliferation, differentiation, and apoptosis. Current models suggest that CBP is present in limiting amounts and transcriptional regulation may be, in part, achieved through competition for this cofactor, as only partial suppression of CBP is required for a phenotype in the CBP heterozygote "knock out" mouse (Kawasaki, et al., 1998; Shang, et al., 2000; Yao, et al., 1998). Consistent with observations in our *in vitro* system, partial suppression of CBP protein levels in virgin CBP(+/-) heterozygote mice results in a 90% incidence of severe mammary gland hyperplasia and hyperlactation (Yao, personal communication). Taken together these observations provide evidence that partial suppression of CBP protein expression promotes 1) loss of growth regulation and polarity and 2) apoptosis resistance.

In this report, we demonstrate that suppression of CBP protein expression results in loss of laminin-5 expression. Laminin-5 is a major component of mammary gland extracellular matrix and loss of laminin-5 immunostaining at the epithelial-stromal interface has been observed in premalignant breast lesions (Henning, et al., 1998, Mercurio, et al., 2001). However, the regulation of laminin-5 gene transcription in normal mammary epithelial tissue and subsequent loss of laminin-5 expression during mammary carcinogenesis is poorly understood (Miller, et al., 2001).

AP-1 response elements are *cis*-acting DNA sequences known to regulate a wide range of cellular processes, including proliferation, apoptosis, survival, and differentiation (Shaulian and Karin, 2002). Despite extensive study, the target genes regulated by AP-1 that mediate these activities have not been completely characterized (Shaulian and Karin, 2002). The transcriptional activity of AP-1 is extremely complex and is thought to be regulated by several different mechanisms. First, AP-1 activity is determined by the composition of the AP-1 heterodimer. Different AP-1 heterodimers display different affinities for a given response element (Metz, et al., 1994; McBride and Damer, 1998; Cook, et al., 1999). Second, posttranslational modifications exert a strong control on AP-1 activity. Notably, phosphorylation of c-jun at Ser 63 and Ser 73 increases its affinity for CBP (Bannister, et al., 1995). Third, AP-1 activation/deactivation may depend on interactions with specific transcriptional co-activators and co-repressors (Karin, 1995; Karin, et al., 1997; Pessah, et al., 2001; Benkoussa, et al. 2002).

CBP is known to interact with the AP-1 response element (Horvai, et al., 1997; Benkoussa, et al. 2002). Several independent approaches, involving co-transfection assays or microinjection of anti-CBP antibodies, have demonstrated that CBP participates in activation of AP-1 (Kwok, et al., 1994; Arias, et al., 1994). Activation of nuclear receptors during steroid/thyroid hormone signaling results in loss of AP-1 activity through recruitment of CBP to the nuclear receptor co-activator complex (Kamei, et al., 1996). It has also been

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recently shown that retinoic acid receptors inhibit AP-1 activity through regulating extracellular signal-regulated kinase and CBP recruitmentment to an AP-1-resposive promoter (Benkoussa, et al. 2002).

The relationship between CBP, AP-1 activity, and laminin-5 expression is poorly defined. Both the mouse and human *LAMA3A* promoter contain an AP-1-"rich" region (Virolle, et al., 1998; Miller, et al., 2001). The second AP-1 binding site present in both the mouse and human *LAMA3A* promoter is critical for baseline transcription of laminin-5 α3-chain (Virolle, et al., 1998; Miller, et al., 2001). Here we show that suppression of CBP results in loss of *LAMA3A* promoter activity and laminin-5 α3-chain expression and 2) blocks the apoptotic elimination of acutely damaged HMEC-E6 cells. This decreased production of laminin 5-α3 correlates with loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Taken together, these observations suggest that 1) CBP occupancy of the *LAMA3A* promoter promotes laminin-5 α3-chain expression and 2) loss of CBP occupancy inhibits laminin-5 α3-chain expression and may promote survival of acutely "damaged" HMECs.

In contrast to our observation that suppression of CBP inhibits laminin-5 expression in HMECs, it has been previously observed that overexpression of the related co-activator, p300, inhibits laminin-5 production in MCF-10A cells (Miller, et al., 2000). One potential explanation for these seemingly divergent results may lie in differences in cell type. MCF-10A is an immortalized human breast epithelial cell line that exhibits complex chromosomal rearrangements (Yoon, et al., 2002). Our CBP suppression studies were performed in either early passage HMEC-LXSN control cells or in early passage HMEC-E6 cells. These transduced cell strains are not immortalized and previous cytogenetic analysis demonstrates the absence of chromosomal rearrangements in early passage transduced HMECs (Seewaldt, et al., 2001a). It is also possible that the difference between these previous studies and our results can be accounted for by differences between CBP and p300

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activities. While p300 and CBP have many overlapping functions, there is ample evidence that they also have distinct activities. For example, CBP and p300 play a distinct role during retinoic acid-induced differentiation in F9 cells (Kawasaki, et al., 1998; Ugai, et al., 1999) and p300, but not CBP, has been shown to be transcriptionally regulated by BRCA1 in breast cancer cell lines (Fan, et al., 2002).

In summary, observations in our model system predict that a partial reduction of CBP expression results in 1) loss of CBP occupancy of the AP-"rich" region of the *LAMA3A* promoter, 2) decreased *LAMA3A* promoter activity, and 3) reduced expression of laminin-5 α3-chain protein. We also observe that loss of CBP/laminin 5-α3 expression blocks rECM-growth regulation, -polarity, and -apoptosis *in vitro* and thereby may promote the clonal expansion of "damaged" HMECs *in vivo*. These observations have potential clinical implications and suggest that suppression of CBP may promote mammary hyperplasia and also may increase the risk of subsequent breast cancer.

Acknowledgments

Footnotes:

[1] Abbreviations: **ECM**, extracellular matrix; **rECM**, reconstituted extracellular matrix; **HMEC**, human mammary epithelial cells; **ER**, estrogen receptor; **HPV-16**, human papillomavirus type 16; **PBS**, phosphate buffered saline; **ECL**, enhanced chemiluminescent detection; **FACS**, fluorescent activated cell sorting; **RT**, room temperature; **SKY**, spectral karyotyping; **DAPI**, 4,6-diamino-2-phenyliodole; **Ab**, antibody; **ODN**, oligonucleotides; **CBP**, CREBP binding protein.

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Table 1: CBP-specific antisense ODN sequences

Target gene	Sequences	Size	Status
CBP			
A3342V	5'-CACTTCAGGTTTCTTTTCATCC -3'	22 bp	Active
A2172Z	5'-CTTCTAGTTCTTTTTGTATCTTGTAG-3'	26 bp	Inactive
scrA3342V	5'- ATTCTCATCATCGTCTTCGTTC-3'	22 bp	Inactive

The first and last three base pairs of each ODN sequence were phosphorothiolate modified.

Table 2: Laminin and integrin primers

Gene	Primer set	Cycle conditions	PCR cycle number
ITGA3	F: 5'-AAGCCAAGTCTGAGACT -3' R: 5'-GTAGTATTGGTCCCGAGTCT -3'	94°C 3 min. 94°C 30 sec. 60°C 1 min. 72°C 1 min. 72°C 7 min.	22
ITGB1	F: 5'-GCGAAGGCATCCCTGAAAGT -3' R: 5'-GGACACAGGATCAGGTTGGA -3'	94°C 3 min. 94°C 30 sec. 54°C 30 sec. 72°C 1 min. 72°C 7 min.	19
LAMA3	F: 5'-TGTGGATCTTTGGGGCAG-3' R: 5'-TTGCCATAGTAGCCCTCCTG -3'	94°C 3 min. 94°C 30 sec. 58°C 30 sec. 72°C 1 min. 72°C 7 min.	20
LAMB3	F: 5'-TGAGGTTCAGCAGGTACTGC -3' R: 5-TAACTGTCCCATTGGCTCAG -3'	95°C 3 min. 95°C 1 min. 55°C 1 min. 72°C 1 min. 72°C 7 min.	23
LAMC2	F: 5'-CTGAGTATGGGCAATGCCAC -3' R: 5'-GCTCTGGTATCAACCTTCTG -3'	95°C 3 min. 95°C 1 min. 55°C 1 min. 72°C 1 min. 72°C 7 min.	22
Beta-actin	F: 5'-GCTCGTCGTCGACAACGGCTC-3' R: 5'-CAAACATGATCTGGGTCATCTTCTC-3' (Invitrogen)	94°C 2 min. 94°C 15 sec. 55°C 30 sec. 72°C 30 sec. 72°C 7 min.	18

Figure 1: Partial karyotypes of a representative late passage HMEC-E6 (passage 20) mitotic cells demonstrate two copies of an unbalanced translocation between chromosomes 13 and 16 involving 16p13 (arrows). *(a)* SKY in display colors (blue, chromosome 13 material; bluish gray, chromosome 16 material). *(b)* SKY in classification colors (red, chromosome 13 material; orange, chromosome 16 material). *(c)* Inverted and contrast-enhanced DAPI image of the same metaphase cels.

Figure 2: *(a)* Expression of endogenous p53 and exogenous HPV-16 E6 mRNA in HMECs. Passage 10 and 18 HMEC-P parental cells **(Parental)**, HMEC-LXSN controls **(LXSN)**, and HMEC-E6 cells **(E6)** were analyzed for p53 and HPV-16 E6 mRNA expression. Ten micrograms of RNA were loaded per lane. 36B4 served as a loading control.

(b) Expression of p53 protein is suppressed in HMEC-E6 cells. Passage 10 and 18 HMEC-P parental cells **(Parental)**, HMEC-LXSN controls **(LXSN)**, and HMEC-E6 cells **(E6)** were analyzed for p53 protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin serves as a loading control.

(c) CBP protein expression is decreased in apoptosis-resistant late passage HMEC-E6 cells. Early and late passage HMEC-LXSN vector controls **(LXSN)** (passages 11 and 16) and HMEC-E6 cells **(E6)** (passages 11 and 18) were analyzed for CBP protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin was used as a loading control.

(d) CBP protein expression is suppressed by antisense ODNs. HMEC-LXSN vector controls **(LXSN)** (passage 12) and early passage HMEC-E6 cells **(E6)** (passage 12) were cultured in the presence of **(1)** no treatment, **(2)** active CBP-specific ODN (A3342V), and **(3)** inactive CBP ODN (scrA3342V). Resultant cells were analyzed for CBP protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin was used as a loading control.

Figure 3: Inhibition of CBP expression in HMECs by antisense ODNs results in enhanced proliferation in rECM. The mean diameter of spheres formed by early passage HMEC-LXSN vector controls (passage 10) *(a)* and early passage HMEC-E6 cells (passage 11) *(b)* treated with either CBP antisense ODN (A3342V) **(CBP-as)** or inactive CBP ODN (scrA3342V) **(CBP-scr)** were plotted as a function of days in culture. Cells were plated in rECM on Day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. Ki-67 *(c, d)* staining indices in early passage HMEC-LXSN cells (passage 10) *(c)* and early passage HMEC-E6 cells (passage 11) *(d)*. Two hundred cells were surveyed per time point and indices were calculated from an average of 3 separate experiments. *Error bars* show standard error.

Figure 4: Suppression of CBP expression in HMECs inhibits polarized expression of E-cadherin and ZO-1 in rECM culture. Localization of E-cadherin and ZO-1 in HMECs treated with CBP antisense ODNs using immunofluorescence microscopy. Frozen section of early passage HMEC-LXSN vector controls (passage 11) *(a, c, e, g)*, HMEC-E6 cells (passage 11) *(b, d, f, h)*, treated with either CBP antisense ODN (A3342V) *(a, b, e, f)* or inactive CBP ODN (scrA3342V) *(c, d, g, h)* grown in rECM for 6 days, cryosectioned, and stained with a monoclonal antibody to E-cadherin *(a-d)* or ZO-1 *(e-h)* as described in Materials and Methods. E-cadherin was localized primarily at points of cell-cell contact in HMEC-LXSN and HMEC-E6 cells treated with inactive CPB ODNs (scrA3342V) *(c, d, g, h* arrows*)*. In contrast, HMEC-LXSN and HMEC-E6 cells treated with antisense CPB ODNs (A3342V) showed dispersed membrane and intracellular staining of both E-cadherin and ZO-1 *(a, b, e, f,* arrowheads*)*.

Figure 5: Inhibition of CBP in early passage HMECs by antisense ODNs blocks apoptosis in rECM culture. Electron micrographs of early passage HMEC-LXSN control cells (passage 11) *(a)* and early passage HMEC-E6 cells (passage 11) *(b)* treated with CBP antisense (A3342V) ODN and grown in rECM for 9 days. Cells formed large, dense, irregularly shaped multicellular colonies with no central lumen *(a, b)*. In contrast, early passage HMEC-E6 cells (passage 10) treated with inactive CBP ODN (scrA3342V) *(c)* underwent apoptosis when grown in rECM for 7 days as evidenced by 1) nuclear condensation **(n)**, 2) cell shrinkage and separation, and 3) margination of chromatin **(mr)**. Percent of apoptotic cells in early passage HMEC-E6 cells (passage 11) *(d)* and early passage HMEC-LXSN controls (passage 11) *(e)* treated either with active (A3342V) or inactive (scrA3342V) CBP-specific ODNs. Apoptosis was measured by TUNEL-staining as described in Materials and Methods. Apoptotic index was measured by calculating the percentage of TUNEL-staining cells relative to the total number of cells surveyed. Data represents an average of three separate experiments. *Error bars* show standard error.

Figure 6: Laminin-5 α3-chain mRNA and protein expression is decreased in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells.

(a) Analysis of differential gene expression in early **(E6E)** and late **(E6L)** passage HMEC-E6 cells (passage 10 and 18) relative to early passage HMEC-LXSN controls (passage 10) **(LXSN)**. Cells were grown in contact with rECM and harvested for differential gene expression as described in Materials and Methods. All RNA combinations used for array analysis were obtained from cells that were matched for passage number, cultured under the identical growth conditions, and harvested at identical confluency. Data was collected in triplicate using independent biological replicates. Array images were processed using Affymetrix MAS 5.0 software as described in Materials and Methods. Pair-wise "treatment vs control" comparisons were made employing CyberT (Baldi, et al., 2001), a Bayesian t-statistic algorithm derived for microarray analysis. Color-coding: green, downregulation of gene expression; red, induction; black, no significant change; grey, no data available.

(b) Semiquantitative RT-PCR analysis of integrin and laminin-5 mRNA expression in early and late passage HMEC-E6 cells (passage 10 and 18) and HMEC-LXSN controls (passage 10 and 16). Expression was normalized to beta-actin. These data are representative of three separate experiments.

(c) Quantitation of RT-PCR expression data. Expression studies were performed in triplicate. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software. Expression was normalized to beta-actin.

(d) Laminin-5 α3-chain protein expression is decreased in rECM-resistant, late passage HMEC-E6 cells (passage 18) relative to rECM-sensitive, early passage HMEC-E6 cells (passage 10) and early and late passage HMEC-LXSN controls (passage 11 and 16). Western analysis was performed as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin serves as a loading control.

Figure 7: Immunofluorescence characterization of α3/β1-integrin and laminin-5 expression in rECM-sensitive and –resistant cells. Frozen section of early passage HMEC-LXSN controls (passage 10) *(a-c),* late passage HMEC-controls (passage 16) *(d-f),* early passage HMEC-E6 cells (passage 10) *(g-i),* and late passage HMEC-E6 cells (passage 18) *(j-l)* grown in rECM for 6 days, cryosectioned, and immunostained for localization of α3-integrin *(a, d, g, j),* β1-integrin *(b, e, h, k),* and laminin-5 α3-chain *(c, f, i, l).* α3-integrin, β1-integrin, and laminin-5 α3-chain expression was primarily localized at the basal surface of early and late passage HMEC-LXSN and early passage HMEC-E6 cells *(a-i).* In contrast, apoptosis resistant, late passage HMEC-E6 cells showed dispersed membrane and intracellular staining of α3-integrin and laminin-5 α3-chain (arrowheads).
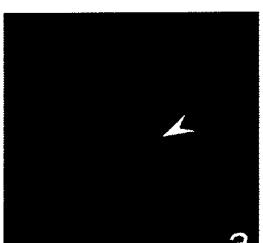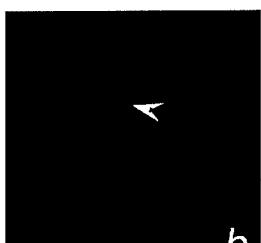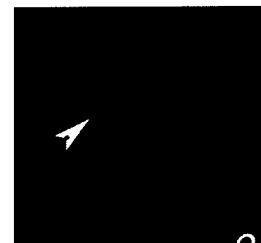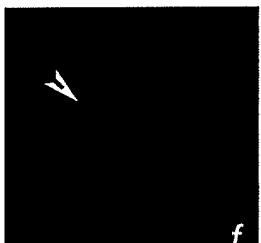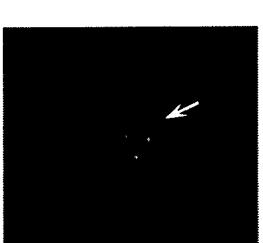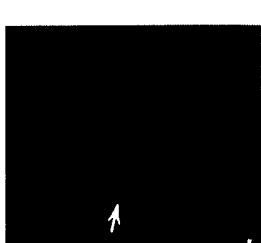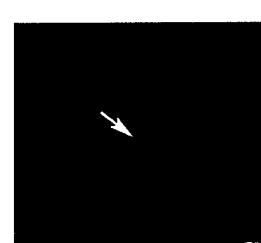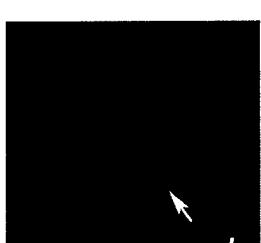
Figure 8: Immunofluorescent characterization of α3β1-integrin and laminin-5 expression in HMECs treated with CBP antisense ODNs. Frozen section of early passage HMEC-LXSN vector controls (passage 11) and HMEC-E6 cells (passage 11) treated either with CBP antisense ODN (A3342V) or inactive CBP ODN (scrA3342V). Cells were grown in rECM for 6 days, cryosectioned, and immunostained for either α3-integrin *(a-d)*, β1-integrin *(e-h)*, or laminin-5 α3–chain *(i-l)* as described in Materials and Methods. α3- and β1-integrin and laminin-5 α3-chain expression was primarily localized at the basolateral surface in HMEC-LXSN and HMEC-E6 cells treated with inactive CBP ODNs *(arrow heads)*. In contrast, HMEC-LXSN and HMEC-E6 cells treated with antisense CBP ODNs showed cells demonstrated disorganized membrane and cytosolic staining of α3-integrin and laminin-5 α3-chain *(arrows)*.

Figure 9: Suppression of CBP expression results in a decrease in *LAMA3A* promoter activity in cells grown in contact with rECM. *(a) LAMA3A* promoter activity was measured in 1) early and late passage HMEC-LXSN controls (passage 11 and 16) and 2) early passage HMEC-E6 cells (passage 10) and compared to rECM-resistant, CBP-"poor" late passage HMEC-E6 cells (passage 18). *(b) LAMA3A* promoter activity is measured in early passage HMEC-LXSN controls (passage 11) or HMEC-E6 cells (passage 11) treated with either 1) CBP-specific antisense ODNs (A3342V) or 2) inactive ODNs (scrA3342V) and grown in rECM. *LAMA3A* promoter activity was measured as described in Materials and Methods. Data represent two experiments performed in triplicate. *Error bars* show standard error.

Figure 10: Suppression of CBP in HMECs grown in rECM promotes decreased occupancy of the 277 bp AP-1-"rich" region of the *LAMA3A* promoter. *(a)* ChIP was performed in 1) early and HMEC-LXSN controls (passage 11 and 16) and 2) early passage HMEC-E6 cells (passage 11) and compared with rECM-resistant, CBP-"poor", late passage HMEC-E6 cells (passage 18). *(b)* Early passage HMEC-E6 cells (passage 10) treated with CBP-specific ODNs, and grown in contact with rECM, were tested by ChIP to determine whether suppression of CBP expression resulted in a loss of CBP-binding AP-1-"rich" site of the *LAMA3A* promoter. ChIP was performed as described in Materials and Methods. Input controls test the integrity of the DNA samples. These data are representative of three separate experiments.

|	
a	**13** **16**
b	**13** **16**
c	**13** **16**

a

HMEC

Parental		LXSN		E6	
p11	p18	p11	p18	p11	p18

p53

E6

36B4

c

HMEC

LXSN p11	E6 p11	LXSN p16	E6 p18

CBP

actin

b

HMEC

Parental		LXSN		E6	
p11	p18	p11	p18	p11	p18

p53

actin

d

HMEC

LXSN			E6		
1	2	3	1	2	3

CBP

actin

	E-cadherin		ZO-1	
	HMEC-LXSN	HMEC-E6	HMEC-LXSN	HMEC-E6
CBP-as	*a*	*b*	*e*	*f*
CBP-scr	*c*	*d*	*g*	*h*

25 μm

HMEC-LXSN AS *a*

HMEC-E6 AS *b*

HMEC-E6 Sen *c*
n
mr

10 μm

d
E6 p11

Percent TUNEL positive cells

■ 5 days
■ 7 days
□ 9 days
■ 11 days

CBP-as   CBP-scr

e
LXSN p11

Percent TUNEL positive cells

■ 5 days
■ 7 days
□ 9 days
■ 11 days

CBP-as   CBP-scr

a.

M10277. Human cytoplasmic beta-actin gene, complete cds
M35198. Human integrin B-6 mRNA, complete cds
U31201. Human laminin gamma2 chain gene (LAMC2), Human laminin gamma2 chain
L34155. Homo sapiens laminin-related protein (LamA3) mRNA, complete cds
X53587. Human mRNA for integrin beta 4
U17760. Human laminin S B3 chain (LAMB3) gene
U31201. Human laminin gamma2 chain gene (LAMC2), Human laminin gamma2 chain
M14648. Human cell adhesion protein (vitronectin) receptor alpha subunit
J03925. Human Mac-1 gene encoding complement receptor type 3, CD11b
X53002. Human mRNA for integrin beta-5 subunit
J05633. Human integrin beta-5 subunit mRNA, complete cds
U40279. Human beta-2 integrin alphaD subunit (ITGAD) gene, exons 25-30
S80335. Integrin beta 7 subunit [human, mRNA, 2798 nt]
X68742. H.sapiens mRNA for integrin, alpha subunit
X64072. H. sapiens CD18 exon 2
M34189. Integrin Beta 1
L25851. Homo sapiens integrin alpha E mRNA, complete cds
M34344. Human platelet glycoprotein IIb (GPIIb) gene
M14199. Human laminin receptor (2H5 epitope) mRNA, 5 end
X07979. Human mRNA for fibronectin receptor beta subunit
U40282. Human integrin-linked kinase (ILK) mRNA, complete cds
D25303. Human mRNA for integrin alpha subunit, complete cds
L36531. Homo sapiens integrin alpha 8 subunit mRNA, 3 end
Y00796. Human mRNA for leukocyte-associated molecule-1 alpha subunit
X16983. Human mRNA for integrin alpha-4 subunit
X02761. Human mRNA for fibronectin (FN precursor)
M61916. Human laminin B1 chain mRNA, complete cds
M59911. Human integrin alpha-3 chain mRNA, complete cds
S70348. Integrin beta 3 {alternatively spliced, clone beta 3C}
X79683. H.sapiens LAMB2 mRNA for beta2 laminin.
X74295. H.sapiens mRNA for alpha 7B integrin
M55210. Human laminin B2 chain (LAMB2) gene
Z26653. H.sapiens mRNA for laminin M chain (merosin)
M15395. Human leukocyte adhesion protein (LFA-1/Mac-1/p150,95 family)
J02963. Human platelet glycoprotein IIb mRNA, 3 end
X02761. Fibronectin. Alt. Splice 1
X06256. Human mRNA for fibronectin receptor alpha subunit
S78569. laminin alpha 4 chain [human, fetal lung, mRNA, 6204 nt]
U33880. Human beta 1 integrin isoform D (ITGB1) gene, partial cds.
X53586. Integrin alpha 6 (or alpha E) protein gene extracted from Human mRNA
U43901. Human 37 kD laminin receptor precursor/p40 ribosome associated protei
M73780. Human integrin beta-8 subunit mRNA, complete cds
M35999, Human platelet glycoprotein IIIa (GPIIIa) mRNA, complete cds

b.

HMEC

LXSN	E6
E L	E L

integrin-α3

integrin-β1

laminin-α3

laminin-β3

laminin-γ2

β-actin

c.

* p ≤ 0.01

Legend:
- LXSN p10
- LXSN p16
- E6 p11
- E6 p18

Y-axis: Relative expression (1 – 1000)

X-axis categories: integrin-α3, integrin-β1, laminin-α3, laminin-β3, laminin-γ2

d.

LX p10	E6 p10	LX p18	E6 p18

laminin-5

actin

	α3-integrin	β1-integrin	laminin-5(α3)
LXSN p10	a	b	c
LXSN p16	d	e	f
E6 p10	g	h	i
E6 p18	j	k	l

25 μm

α3-integrin

	HMEC-LXSN	HMEC-E6

CBP-as

CBP-scr

β1-integrin

	HMEC-LXSN	HMEC-E6

CBP-as

CBP-scr

25 μm

Laminin-5 (α3)

	HMEC-LXSN	HMEC-E6

CBP-as

CBP-scr

25 μm

a

b

a

HMECs

Input Control				laminin-5-α3 ab			
LXSN		E6		LXSN		E6	
p11	p16	p11	p18	p11	p16	p11	p18

b

HMEC-E6

Input Control			CBP ab		
C	Scr	As	C	Scr	As